THE ROLE OF HYPOXIA INDUCIBLE FACTOR (HIF)-1/2α IN THE INFLAMMATORY RESPONSE TO INHALED ALLERGENS

By

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ABSTRACT

THE ROLE OF HYPOXIA INDUCIBLE FACTOR (HIF)-1/2α IN THE INFLAMMATORY RESPONSE TO INHALED ALLERGENS

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Allergic airway disease is characterized by a T helper type 2 cell-mediated airway inflammation and airway hyperresponsiveness. Little is known about the role of hypoxia-mediated signaling in the progression of the disease. To address this knowledge gap, a mouse model was created in which doxycycline exposure induces the functional deletion of hypoxia inducible factor-1α, 2α, or 1/2α from alveolar type II and Clara cells of the lung. When hypoxia inducible factor-1α deletion was induced during the early post-natal development period of the lung, the mice displayed an enhanced response to the ovalbumin model of allergic airway disease. These hypoxia inducible factor-1α-deficient mice exhibit increased cellular infiltrates, eosinophilia in the lavage fluid and parenchyma, and T helper type 2 cytokines, as compared with ovalbumin-treated control mice. Moreover, these hypoxia inducible factor-1α–deficient mice displayed increased airway resistance when compared to their control counterparts. Interestingly, if the loss of hypoxia inducible factor-1α was induced in early adulthood, the exacerbated phenotype was not observed. When the HIF2α deletion was induced during the early post-natal development period of the lung, the HIF2α-deficient mice were not significantly different from their HIF2α-sufficient counterparts. When the HIF1/2a deletion was induced in the early post-natal development period of the lung, the HIF1/2a-deficient mice seemed to be somewhat protected from the increase in cellular

infiltrates and eosinophils. Taken together, these results suggest that epithelial hypoxia inducible factor-1α plays an important role in establishing the innate immunity of the lung, and epithelial-specific deficiency in the transcription factor during early post-natal development increases the severity of inflammation and functional airway resistance, following ovalbumin challenge. Also, the balance between HIF1α and HIF2α levels seems to be important when mounting an immune response. Hyperoixa experiments were also used to aid in identification of potential epithelial signaling factors responsible for the changes in immunity. Different populations of dendritic cells were examined via flow cytometry to determine if they were different after loss of HIF1α. There was a slight increase in plasmacytoid dendritic cells in HIF1α-deficient lungs. These results might explain some of the chronic respiratory pathology observed in premature infants, especially those who receive supplemental oxygen. This early hyperoxic exposure, from normal ambient and supplemental oxygen, would presumably inhibit normal hypoxia inducible factor-1α signaling, mimicking the functional deletion described.

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He has offered unending advice and encouragement while helping me keep everything in perspective and for that I am deeply grateful.

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KEY TO SYMBOLS OR ABBREVIATIONS

αKG	alpha ketoglutarate
AB/PAS	alcian blue/Periodic Acid Schiff
a	alveolus
ad	alveolar duct
ARNT	aryl hydrocarbon receptor nuclear translocator
ATII	alveolar type II
BALF	bronchoalveolar lavage fluid
bHLH	basic helix-loop-helix
BPD	bronchopulmonary dysplasia
BSA	bovine serum albumin
bv	blood vessel
CO_2	carbon dioxide
CoQ	ubiquinone
DC	dendritic cell
DOX	doxycycline
DMOG	dimethyloxyalylglycine
CTAD	carboxy transactivation domain
Eos	eosinophil
EPA	Environmental Protection Agency
EPAS1	endothelial PAS domain protein 1
EPO	erythropoietin

ETC	electron transport chain
FACS	fluorescence activated cell soring
FBS	fetal bovine serum
Fe	iron
Glut1	glucose transporter 1
GMCSF	granulocyte macrophage colony stimulating factor
H&E	hematoxylin and eosin
H^+	protons
HIF	hypoxia inducible factor
HRE	hypoxia response element
i.p.	intraperitoneal
iTreg	induced regulatory T cell
INF	interferon
IgA	immunoglobulin A
Ikaros	Ikaros family zinc finger protein 1
IL	interleukin
Io	ionomycin
KC	keratinocyte chemoattractant
Lymph	lymphocytes
Macs	macrophages
MBP	major basic protein
MCM	mucus cell metaplasia
mDC	myeloid dendritic cell

mmHg	millimeters of mercury
NK	natural killer
NTAD	amino transactivation domain
nTreg	naturally occurring regulatory T cell
ODD	oxygen dependent degradation
OVA	ovalbumin
PAS	PER/ARNT/SIM
pDC	plasmacytoid dendritic cell
PHD	prolyl hydroxylase domain
PMA	phorbol 12-myristate 13-acetate
PMN	neutrophil
PN	post natal
pO_2	partial pressure of oxygen
QH_2	ubiquinol
ROS	reactive oxygen species
rtTA	reverse tetracycline transactivator
SEM	standard error of the mean
SIM	simple minded
TAM	Tamoxifen
tb	terminal bronchiole
T cells	T lymphocytes
TCA	tricarboxylic acid
Th	T helper

TNF	tumor necrosis factor
Treg	regulatory T cell
TSLP	thymic stromal lymphopoitin
UBC	ubiquitin C
ULAR	University Laboratory Animal Resource
VEGF	vascular endothelial growth factor
VHL	von Hippel-Lindau

CHAPTER ONE

Introduction

1.1 Overview of lung anatomy and physiology

The respiratory system is made up of several organs including the nasal cavity, pharynx, larynx, trachea, bronchi and lungs. The lung is the main organ, and its primary function is gas exchange replacing expired air containing carbon dioxide with inspired air containing oxygen. The inspiration of the oxygen necessary for survival comes with the potential risk of inhaling particles, chemicals, and microorganisms. This direct contact with the environment requires that the lungs be equipped with special defenses. The lungs have evolved a complex structure, both at a tissue level and at a cellular level, to be able to carry out gas exchange while keeping contaminants from causing undue inflammation.

In the mouse, there are five total lung lobes, four on the right and one continuous lobe on the left. There are two main sections of the respiratory airways, the conducting zone (upper airways) and the respiratory zone (lower airways). In the mouse, the conducting zone is made up of the nasal cavity, pharynx, larynx, and trachea, while the respiratory zone is made up of the terminal bronchioles, alveolar ducts, and alveoli. In other mammals, such as humans, respiratory bronchioles are also found in the respiratory zone, however mice, as well as, rats and horses, do not have this type of bronchioles. Both the upper and lower airways have anatomical barriers that help protect the lungs from foreign particles. Notably, these include the cough reflex and the mucociliary elevator containing enzymes and immunoglobulin A (IgA).

The conducting zone, also known as the upper airways, is the part of the respiratory tree that is located outside of the lung proper, beginning with the nasal cavity. There are three pairs of turbinates located in the mouse nasal cavity. Each of

these turbinates is surrounded by respiratory mucosa consisting of two layers: a highly vascularized and innervated lamina propria covered by respiratory epithelium [1]. The turbinates are known for having a high surface area, aiding in warming and moistening the air prior to its entry into the lungs. The pharynx is the next structure in the conducting airways. It is located behind the nasal and oral cavities as well as the larynx [2]. The pharynx has openings into all three of these areas; therefore air, fluids, as well as food, all passes through the pharynx. The particular portion of the pharynx that air passes through is called the nasopharynx. Since mice are obligate nose breathers, absolutely all inspired air in the mouse passes through the nasopharynx on its way to the lungs. Next in the conducting portion of the respiratory tract is the larynx. The larynx is a structure shaped like a tube that joins together the previously mentioned pharynx with the next structure, the trachea. The larynx has several functions including keeping the airways open during respiration and closing the airway when food or fluids are being swallowed. Additionally, the larynx must also be structurally sturdy so that when air enters the lungs the conduit is not collapsible due to the negative pressure [3]. Finally, the trachea is formed by C-shaped rings of cartilage and, in the mouse, is lined with respiratory mucosa. There are three main cell types that make up the epithelial lining of the mucosa: nonciliated secretory (Clara cells and goblet cells), ciliated, and basal cells. Typically, fifty to sixty percent of the cells are nonciliated Clara cells [4]. This statistic is unique to mice, as Clara cells are only found in the terminal bronchioles of most species [5]. In addition, Clara cells have cytochrome P-450 monooxygenases which result in these cells being susceptible to injury from many pulmonary as well as circulating toxicants [6, 7].

The respiratory zone is where gas exchange actually occurs. In mice, this is made up of the terminal bronchioles, alveolar ducts, alveolar sacs and alveoli. The airway epithelium in the terminal bronchioles is mostly comprised of the nonciliated secretory Clara cellswhich are also found in the conducting zone, and ciliated cells. Just like the Clara cells residing in the mouse trachea, these Clara cells also contain cytochrome P-450 monooxygenases which metabolize and detoxify inhaled toxicants [7]. The alveolar ducts, alveolar sacs and alveoli are the gas exchange unit of the lung. The alveolar epithelium is made up of alveolar type I (93-97% of surface area) and alveolar type II (ATII) (60% of cells, though just 3-5% of surface area) cells [8]. The primary job of the ATII cells is to produce surfactant and its associated proteins while the primary role of the ATI cells is to provide adequate surface area for gas exchange to occur. It should also be noted that alveolar type I cells are incapable of mitosis. Because of this, if they are damaged, they have little to no ability to repair themselves. ATII cells, however, are capable of repair, as well as differentiating and replacing the alveolar type I cells when they are damaged [9].

The lungs are the only organ in the body to receive the entirety of the cardiac output as they are a part of the bronchial circulation as well as the pulmonary circulation. The bronchial circulation is the portion of the greater systemic circulation that serves the metabolic needs of the lung itself. The majority of the blood being supplied to the lungs is via the pulmonary circulation. This portion of the circulation is to facilitate the exchange of carbon dioxide for oxygen by bringing the blood in close contact with the alveoli. The pulmonary circulation carries deoxygenated blood away from the heart and to the lungs where the gas exchange occurs. The freshly

oxygenated blood is then carried back to the heart where it is pumped and distributed systemically.

1.2 Biological role of oxygen

Oxygen is a very important biological molecule, playing a role in energy production and metabolism. During physiological respiration, air is taken into the lungs and the oxygen in the air is traded for carbon dioxide in the blood in the capillaries surrounding the alveoli. Once the oxygen enters the blood, it is bound by hemoglobin, a protein with 4 subunits. Each of the subunits has a heme group associated with it, consisting of an iron (Fe) ion in the middle of a porphyrin ring. The heme in hemoglobin reversibly binds oxygen only when iron is in the Fe^{2+} state. When iron is oxidized to the Fe^{3+} state, heme does not bind oxygen and the hemoglobin molecule is then called methemoglobin. When oxygen is bound to the hemoglobin, the complex is termed 'oxyhemoglobin'. The oxyhemoglobin can then deliver the oxygen to peripheral tissues, using the difference in partial pressure of oxygen (pO_2) between the blood and the peripheral tissue to drive oxygen out of the blood and into the tissue via passive diffusion [10].

In the cells of the peripheral tissues, the mitochondria are the primary site of oxygen utilization. It is there that the electron transport chain (ETC) is located and oxidative phosphorylation takes place. The ETC is made up of four distinct complexes, I, II, III, and IV, embedded in the inner mitochondrial membrane. Each of the complexes carries electrons originally donated by NADH or $FADH_2$ and ultimately transfers them to oxygen. Briefly, at complex I, electrons are transferred from NADH to ubiquinone

(CoQ), reducing it to ubiquinol (QH_2) and resulting in protons (H^+) being pumped into the intermembrane space of the mitochondria. At complex II, succinate from the tricarboxylic acid (TCA) cycle is oxidized to fumarate and electrons are donated to CoQ, resulting in fumarate and QH_2. No H^+ are pumped at this complex, but complex II, along with complex I feed the pool of QH_2. At complex III, the QH_2 donates electrons to cytochrome C, resulting in reduced cytochrome C and the pumping of additional H^+ into the intermembrane space. Complex III is the only complex that is capable of releasing reactive oxygen species (ROS) into the intermembrane space of the mitochondria. Complex IV is where the transfer of electrons from cytochrome C to molecular oxygen takes place. Molecular oxygen is the final electron acceptor of the ETC and is converted into water. All of these preceding steps are required to maintain the H^+ gradient across the inner membrane of the mitochondria which is what drives the H^+-ATPase, converting ADP and P_i to ATP. Approximately 95% of the oxygen exchanged in the lung is consumed as the final electron acceptor at cytochrome c oxidase, Complex IV [11]. Oxygen, therefore, is essential for the efficient production of ATP via the ETC and the loss of oxygen tension, also known as hypoxia, would have a strong negative impact on both cellular energetics and metabolic flux.

1.3 Normoxia and hypoxia

The air we breathe is predominantly made up of nitrogen, but it contains approximately 21% oxygen, as well as smaller amounts of other gases like carbon dioxide. Dalton's Law states that the total pressure of a gas mixture is due to the partial

pressure exerted by each gas in the mixture. Inhaled air, at sea level has a total pressure of 760 mmHg. Once the air is inhaled, it becomes saturated with water vapor in the lungs. Water vapor has a pressure of 47 mmHg, and therefore, using Dalton's Law, the pO_2 of oxygen in the lungs is 150 mmHg. Once the inspired air travels to the alveoli, the pO_2 is altered by the exchange of CO_2 for O_2 across the capillary membrane, as well as by mixing with dead space gas, resulting in an arterial blood pO_2 of approximately 100 mmHg. As the blood carrying oxyhemoglobin travels through the circulatory system, it encounters capillaries in many different tissues. In these capillaries, there is a higher pO_2 in the blood than in the tissue. This disparity in pO_2 causes the oxygen to dissociate from the hemoglobin and enter the tissue via diffusion.

Cells are adapted to the pO_2 that they are routinely exposed to; this state of normal oxygen concentration is termed 'normoxia'. Once the pO_2 in the tissues or cells falls below this normal level, the tissue or cells are considered in a state of hypoxia [12]. Due to the important role of oxygen in the ETC and oxidative phosphorylation, cells have evolved a sensitive response to hypoxia that involves many cellular adaptations, including a metabolic switch to glycolysis (anaerobic metabolism).

It is important to note that hypoxia occurs during many normal physiological events, for example, fetal development. These hypoxic signals drive an adaptive response that is centered on several common processes, such as angiogenesis. In addition to these normal physiological occurrences that involve hypoxia, many pathological conditions like cardiovascular disease, stroke and cancer involve

decreases in available of oxygen [13]. On a cellular level, hypoxia can either lead to cell adaptation or cell death depending on the level of oxygen deficiency.

During hypoxic stress, cells utilize several adaptive mechanisms to cope with and ultimately address the decrease in oxygen availability. One mechanism by which a cell can adapt is an increased rate in glycolytic capacity to compensate for the energy debt the cell encounters because of the decrease in oxidative phosphorylation [14]. To achieve this increase in glycolysis, hypoxia drives the expression of genes that encode glucose transporters, such as Glut1, and glycolytic enzymes, such as hexokinase and phosphofructokinase [15]. To increase the supply of oxygen, hypoxia will also induce the transcription of genes that encode proteins necessary for increased angiogenesis, such as vascular endothelial growth factor (VEGF) [16]. Finally, systemic hypoxic stress increases the kidney's production of erythropoietin (EPO) as well as red blood cell volume [17]. Hypoxia-induced signaling can also impact secondary signaling that might promote adaptation at different levels. For example, hypoxia plays an important role in inflammation [18]. Central to each of these responses to loss of normal oxygen tension is a family of proteins known as the hypoxia inducible factors (HIFs).

1.4 Hypoxia Inducible Factors (HIFs) and hypoxia signaling

The cellular response to hypoxic stress is predominantly regulated by a family of transcription factors sensitive to hypoxia. The most well studied of these transcription factors are the hypoxia inducible factors (HIFs). The HIFs are a family of heterodimeric transcription factors that are members of the basic helix-loop-helix (bHLH)-PER/aryl hydrocarbon receptor nuclear translocator (ARNT)/single minded (SIM) superfamily of

environmental sensors (PER/ARNT/SIM or PAS) [17]. HIFs function as heterodimers and they are made up of an alpha and a beta subunit. The alpha subunits include three isoforms: HIF1α, HIF2 α and HIF3 α, while the beta subunits include ARNT1 (also known as HIF1β) and ARNT2 (Figure 1). Of the three isoforms of the alpha subunit, HIF1α is the most widely studied, is evolutionarily conserved and constitutively expressed in all cell types, and is the major regulator of the cellular hypoxic response [12, 19]. The HIFαs have an N-terminal basic helix-loop-helix (bHLH) domain that is needed for DNA binding. Additionally, the HLH domain is the major dimerization interface. Both of these regions are required for the formation of functional DNA binding complexes [13, 20]. The PAS domain acts as the secondary dimerization interface which can allow binding with a second bHLH/PAS partner (ARNT) in the nucleus.

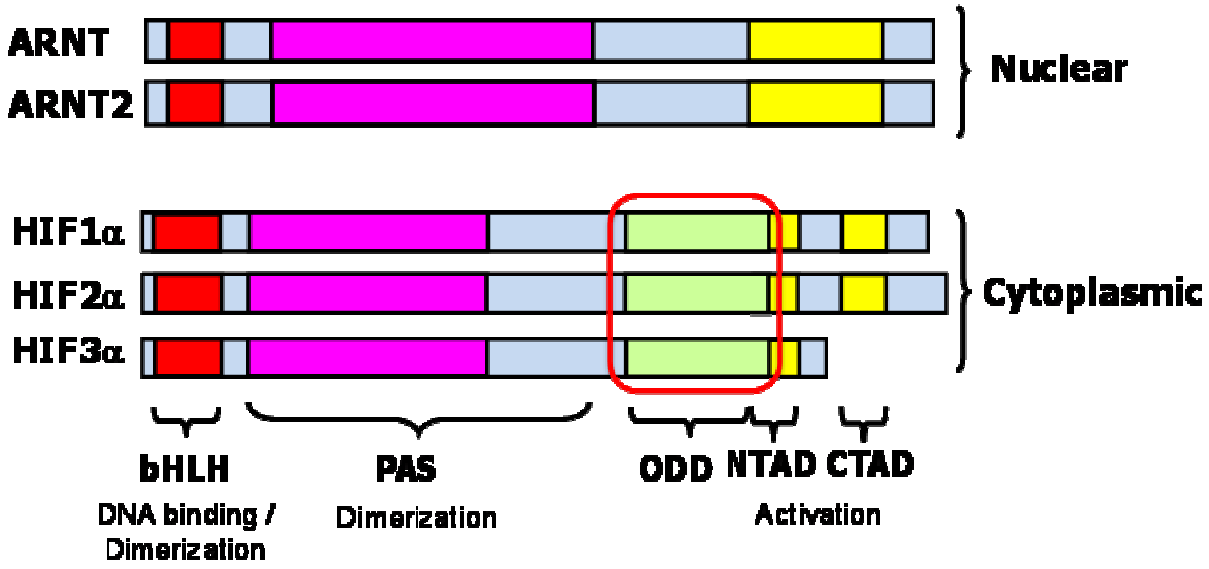


Figure 1 **Protein structure of HIFs**. HIFαs and ARNTs contain an N-terminal basic helix-loop-helix (bHLH) for DNA binding and dimerization. They also contain the Per-Arnt-Sim (PAS) domain for dimerization. HIFαs have an oxygen-dependent degradation domain (ODD). Finally, both HIFαs and ARNTs contain a amino-transactivation domain (NTAD) and a carboxy-transactivation domain (CTAD).

HIFαs are unique because they have two oxygen-dependent degradation (ODD) domains. Because of this, HIFα protein levels are dependent on cellular oxygen and are post-transcriptionally regulated [12, 19].

ARNT and ARNT2 are constitutively expressed and localized to the nucleus. HIFαs are also constitutively expressed, but, in contrast to ARNTs, are localized to the cytoplasm. However, they are quickly degraded in an oxygen-dependent manner that relies on a group of prolyl hydroxylases, known as the prolyl hydroxylase domain (PHD) enzymes. During normoxia, the oxygen-dependent degradation domain (ODD) of the HIFαs is modified by the PHD enzyme. PHDs require four things for function: ferrous iron (Fe^{2+}), ascorbate, α-ketoglutarate (αKG), and oxygen. The hydroxylated ODD is recognized by the von Hippel-Lindau (VHL) protein which, in turn, recruits ubiquitination machinery, targeting HIFαs for proteasomal degradation [21, 22] (Figure 2A). Under hypoxic conditions, the PHDs are unable to hydroxylate HIFs, thus leading to the stabilization of the transcription factor [23, 24]. The unmodified HIFαs are then signaled by an unknown mechanism to translocate to the nucleus where they can bind ARNT, its heterodimeric partner. The HIF/ARNT heterodimer is an active transcription factor which binds to hypoxia response elements (HREs) within genomic DNA. The HIF transcription factor is capable of regulating the expression of more than 150 target genes [13, 25] (Figure 2B).

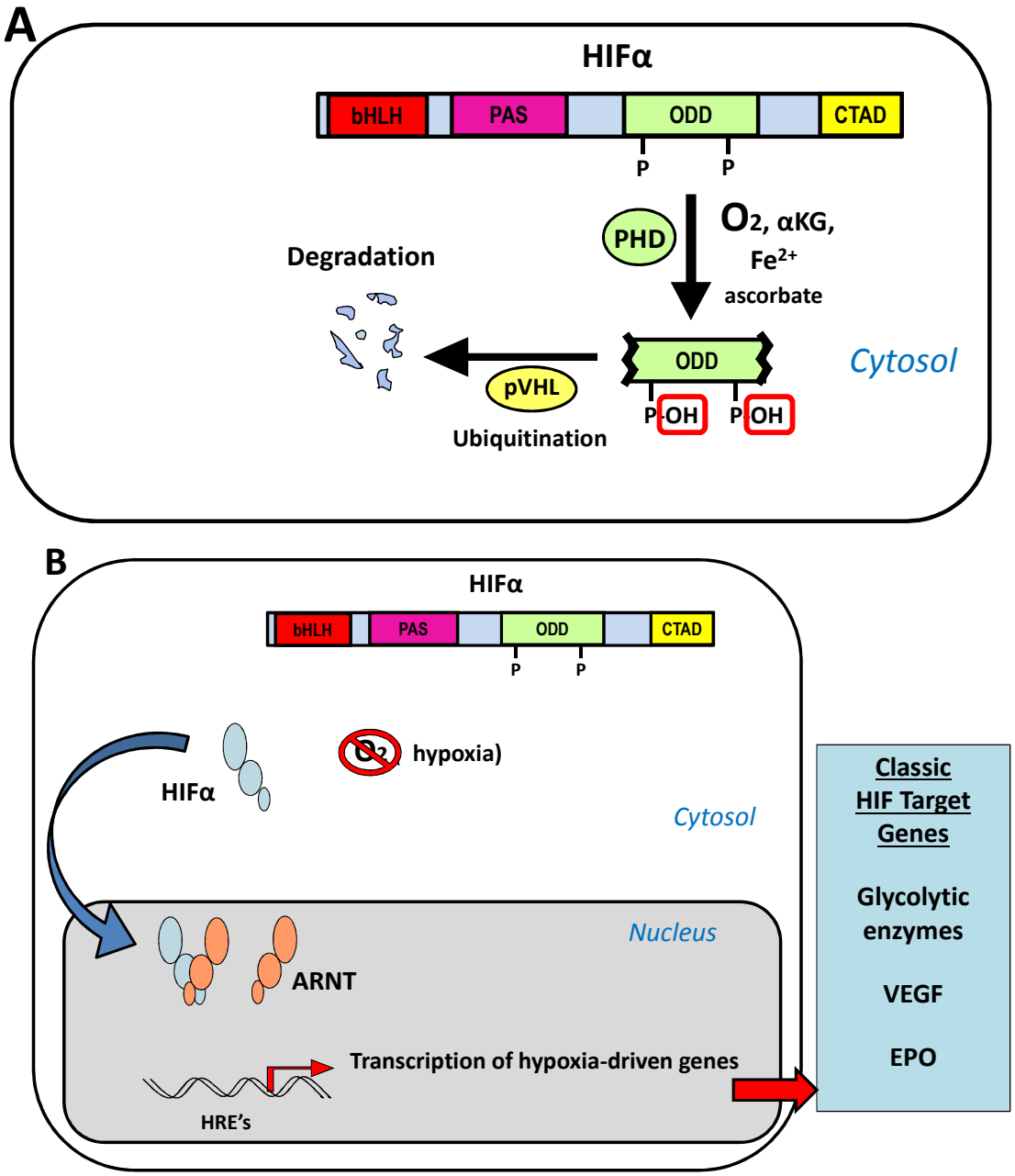


Figure 2 **Oxygen-dependent regulation of HIFα**. HIFα protein is constitutively expressed. At normoxia, prolyl hydroxylase (PHD) uses oxygen to modify proline residues on HIFα. This recruits the von Hippel Lindau (pVHL), an ubiquitin ligase, which targets HIFα for degradation by the proteasome (**A**). In contrast, during hypoxia, HIFα can accumulate in the cytoplasm and translocate to the nucleus where it heterodimerizes with ARNT, forming the functionally competent HIF transcription factor. This complex binds to hypoxia response elements (HRE) on DNA and thereby directs transcription of over 150 genes (**B**).

HIF1 and HIF2 do not completely overlap in their ability to transcriptionally regulate hypoxia responsive genes. It appears that glycolytic genes are predominantly regulated by HIF1 [26], whereas HIF2 has been suggested as the main regulator of vascular endothelial growth factor (VEGF) and erythropoietin (EPO), even in tissues that express both HIF1 and HIF2 [27]. It is also thought that HIF2 and HIF3 might interact with the binding site of HIF1 target genes, allowing for some of the overlap in control [25].

It was originally thought that since there were several HIFs and partially overlapping expression, these may be functionally redundant. Knockout studies in mice have shown that HIF1α and HIF2α have distinct roles. Both HIF1α and HIF2α knockouts were embryonic lethal, however they had different phenotypes. It was shown that HIF1α was needed for mesenchymal cell survival and angiogenesis during embryonic development. These mice die at approximately embryonic day 9.5 from placental, cardiovascular, and neural tube defects [28, 29]. Mice lacking HIF2α, sometimes referred to as endothelial PAS domain protein 1 (EPAS1), showed differing diverse cardiovascular phenotypes. In one genetic background, the organ of Zuckerkandl, a major catecholamine source during development, does not produce enough of the catecholamines, leading to embryonic death, likely due to bradycardia [30]. In a different genetic background, HIF2α played an important role in remodeling the primary vascular network [31]. Finally, another study found HIF2α to be essential for normal development while playing an important role in stem cell biology [32]. When normal tissue was analyzed to determine HIF2α expression, its highest expression was found in bone marrow. Further analysis localized HIF2α expression to the

macrophages of other tissues, notably the lung [33]. Interestingly, HIF3α knockout mice also display cardiovascular abnormalities, as well as, a lung remodeling phenotype [34]. Recently, conditional knockout mouse models have been developed for HIF1α and HIF2α and been used to explore the cell-specific roles of these transcription factors in various processes, including inflammation and immunity.

1.5 Hypoxia and its role in asthma and allergic airway disease

The Environmental Protection Agency (EPA) reports that over 23 million people in the US have asthma, which includes 7 million children. According to EPA estimates, asthma costs the US economy approximately $20 billion annually. Asthma is often described based on its clinical manifestations (ie: wheezing, shortness of breath, chest tightness and coughing) which are linked to exposure to various allergens, second hand smoke and pollution. In addition to the clinical symptoms of the disease, there are several cellular and structural markers of asthma observed in affected lung tissue, such as inflammatory cell recruitment (primarily eosinophils), mucus cell metaplasia, narrowing of the lumen, basement membrane thickening and airway hyperreactivity [35].

Asthma attacks can lead to pulmonary and systemic hypoxia. Lung biopsies from asthmatics have shown increased levels of HIF1α [36]. The role of HIF1α-mediated signaling could directly affect the inflammatory responses seen in the lung tissue of asthmatics, which would provide a link between hypoxia signaling and asthma [37]. Though some information is available regarding the role of HIFs in the asthmatic process, the exact role HIFs play in the development and susceptibility to atopic diseases, such as asthma, remains unknown. Recent research has suggested that loss

of these transcription factors can impact the function of different inflammatory cells, including thymocytes.

1.6 T helper cell differentiation

The thymus is an organ in the immune system. It is at this site where T-lymphocytes (T cells), important in the adaptive immune system, differentiate. Thymocytes are progenitor cells, meaning that they are already more specific than a stem cell and will differentiate into its target cell, in this case lymphocytes, found in the blood [38]. In the thymus, these thymocytes differentiate into mature T cells. There have been several cytokines proposed to control early T cell development, but only interleukin-7 (IL-7) has been shown to be essential. IL-7 stimulates the proliferation of immature thymocytes and mature T cells, as well as B cell progenitors via its interaction with its high-affinity receptor (IL-7R) [39]. IL-7R knockout mice have a significant reduction in thymic and peripheral lymphoid cells, with both T cell and B cell development being blocked at an early stage [40]. Studies have not been able to determine if IL-7R is working alone, or if it is functioning with other cytokines. In addition to cytokines, there have been several transcription factors that have been proposed to have a role in T cell differentiation. Only one of these, Ikaros family zinc finger protein 1 (Ikaros), has been determined to be selective for lymphoid development [38]. During development, Ikaros mRNA can be detected in mouse fetal liver and then later, in the embryonic thymus when the hemopoietic and lymphoid progenitors are beginning to localize to these organs. It has been shown that Ikaros is expressed in the T cells and their progenitors, as well as in early B cells in adult mice [41]. Based on both this specific expression pattern of Ikaros mRNA and the discovery of high affinity

binding sites for Ikaros protein in the regulatory domains of many lymphocyte-specific genes, Ikaros seemed likely to be a major factor in lymphoid lineage commitment. This hypothesis was confirmed when mice homozygous for a germline mutation in the Ikaros DNA-binding domain lacked both mature and immature T and B lymphocytes, as well as natural killer (NK) cells [42].

Effector T cells, also known as helper T (Th) cells are the key cells that steer the immune response. The differentiation of these different types of Th cells is characterized by the production of different cytokines. One of the first Th cells to be described were the Th1 cells. These cells produce their signature cytokine, interferon-γ (IFN-γ), as well as other pro-inflammatory cytokines like tumor necrosis factor-α (TNF-α) and TNF-β which stimulate innate and T cell immune responses. The Th1 cells play an important role in protecting the host from intracellular pathogens. There is evidence that particularly strong pro-inflammatory signaling of the Th1 cells can cause tissue damage as well as the unwanted and self-reactive inflammation as is the case in inflammatory bowel disease [43], insulin-dependent diabetes mellitus [44] and rheumatoid arthritis [45]. Interleukin-12 (IL-12) is the primary cytokine that induces Th1 differentiation [46]. IL-12 signaling then promotes expression of Th1-specific transcription factor, T-bet, which belongs to the T-box family of transcription factors. T-bet is quickly and specifically induced in developing Th1 cells and is critical for initiating Th1 differentiation [47].

T helper type 2 cells were another of the early identified Th cells. Th2 cells are defined as producers of IL-4, IL-5, IL-9, IL-10 and IL-13. Unlike Th1 cells that protect from intracellular pathogens, the Th2 response is important in protection against

extracellular pathogens. Additionally, Th2 cells are important for mucosal immunity, specifically in the lung. Overactivity of Th2 cells typically results in chronic inflammatory airway diseases, such as atopic asthma and allergy [48, 49]. IL-4 is the only cytokine determined essential for Th2 differentiation [50]. As with Th1 cells, there is a transcription factor that is required for Th2 differentiation. In this case, the transcription factor is GATA binding protein 3 (GATA-3) which has been identified as the master regulator for Th2 differentiation [51, 52].

Regulatory T (Treg) cells were discovered after Th1 and Th2 cells, but are actually upstream of, and capable of converting into both of these subsets of helper cells. Tregs can either develop in the thymus, termed naturally occurring Treg, or nTreg, or they can be differentiated from naïve T cells in the presence of TGF-β, termed induced Treg, or iTreg. Treg cells are different from the Th1 and Th2 cells, since they do not promote an immune response. Treg cells are immunosuppressive and are important in maintaining self-tolerance and immune homeostasis, as well as re-establishing immune homeostasis after clearance of a pathogen [53, 54]. The transcription factor specifically associated with and called the master regulator of Treg cells is forkhead box P3, also called Foxp3.

Finally, the other main T helper cell type is Th17, a more recently identified population that plays an important role in the induction and continuation of autoimmunity. Interleukin-17, as well as transforming growth factor-β (TGF-β) and IL-6 have all been identified as working together to promote the Th17 conversion [55, 56]. Retinoic acid-related orphan receptors (ROR) are the important transcription factors involved in Th17 differentiation [57].

The most relevant of the T helper cells to airway inflammation are the Th2 cells. Currently, extensive research is being conducted to better understand how the Th2 cytokines act on resident cells in the lung, including the airway epithelium and smooth muscle to trigger the asthmatic phenotype. There have been several studies that have found increased amounts of activated T cells in the lungs of asthmatics, and many of those studies found that those cells expressed cytokines and transcription factors associated with Th2 cells [45, 49]. Experiments using mouse models have determined the important role that Th2 cytokines have in mucus hypersecretion and airway hyperresponsiveness (AHR), key features in the pathophysiology of asthma. IL4 and IL13, two key Th2 cytokines, plat important roles in allergen-induced airway dysfunction and remodeling [58].

1.7 Dendritic cells and their involvement in allergic airway inflammation

Dendritic cells (DCs) are very important in the initiation and modulation of immune responses. DCs are uniquely suited to this task, since they are present both above and below the basement membrane of the respiratory epithelium [59] where they are constantly sampling the antigens they encounter and reporting to the immune system. DCs are capable of inducing primary lymphocyte responses and have been found to be the main cells involved in directing Th1-mediated responses.

Unlike the case of Th1-mediated inflammation, there is no mechanism known yet for DCs directing Th2-mediated responses, though there are a number of animal studies using the ovalbumin (OVA) sensitization model which hint at the role of DCs in the development of experimental allergic asthma. For example, mice exposed to an allergen, such as ovalbumin, have significant increases in the numbers of DCs in the

airways [60]. In addition, DCs that have been pulsed with OVA are capable of inducing a Th2-mediated inflammation in the lungs once they have been placed into the airways of the animals [61]. Finally, DC depletion from OVA-sensitized mice eliminates the allergen-induced airway hyperreactivity and additionally, the addition of these DCs back in the system will restore the allergic phenotype [62]. Taken together, it would seem that DCs have an important role in not just the development, but also the maintenance of allergen-induced airway inflammation.

There are several subsets of DCs in the lung, each with distinct functions. These populations are determined based upon the cell surface markers that they express. Though reports suggest overlap in these surface markers, using distinct combinations, it is possible to categorize the subsets of DC cells within the tissue. It should be noted that there are many combinations of surface markers that can be used to sort out the populations, and it is sometimes difficult to compare one flow cytometry study to another, due to their differing use of surface markers. In general, there are two major subsets of DCs, myeloid dendritic cells (mDCs) and plasmacytoid dendritic cells (pDCs). There are further subsets that can be identified, but only mDCs and pDCs will be discussed here.

Some mDCs are associated with the respiratory epithelium, projecting their dendritic fingers in between epithelial cells, directly sampling the airway [63]. Some other mDCs are found under the basement membrane in conducting airways and lung parenchyma. These mDCs are able to secrete proinflammatory cytokines, such as TNFα, resulting in attracting Th2 cells to the lung, a very important step in the development of allergic airway inflammation [64].

The other subset of DCs is pDCs which have the ability to drive the development of regulatory T cells (Tregs) [65]. In their ability to drive development of Tregs, pDCs provide protection against inflammatory responses. Studies with mouse models of OVA have shown that depletion of pDCs results in eosinophila as well as Th2 cytokine production when animals are challenged (but not sensitized) with OVA. However if pDCs were replaced in the mice before OVA challenge, there were no hallmarks of allergic airway inflammation [65].

1.8 Epithelial-derived factors capable of influencing immunity

Preliminary data has demonstrated that loss of HIF1α signaling can influence the development of normal immunity in the lung. This suggests that epithelial cells produce factors capable of directing the programming of immunity in the tissue. Recently, more attention has been given to the idea that the epithelium plays a central role in inflammation and immunity [66]. There are several epithelial-derived factors that have been shown to influence immunity, especially in the lung. These factors include galectin 3, thymic stromal lymphopoietin (TSLP), interleukin-1 receptor type II (IL1R2), and granulocyte macrophage colony stimulating factor (GMCSF). Galectins are a family of β-galactoside binding proteins that have intra- and extracellular functions. Several different labs have indicated a role for galectin-3 in inflammation, and more importantly, galectin-3 has even been shown to play a role specifically in OVA-induced airway inflammation [67] and dendritic cell function [68]. Additionally, there is evidence that galectin-3 functions as an adhesion molecule to aid in eosinophil rolling and adhesion [69]. Taken together, there are many indications that galectin-3 is an attractive candidate as a mediator between HIF1α and Th2 bias. Interleukin-1 (IL1) is a cytokine

capable of modulating airway hyperreactivity [70]. Factors that are capable of modulating IL1, therefore, are important in developing normal immunity. IL1R2 blocks processing of IL1β propeptide and binds mature polypeptide, inhibiting its ability to bind IL1R1. The loss of IL1R2 expression should increase the levels of IL1β [71]. TSLP is an epithelial-derived factor that is capable of programming dendritic cells and has been demonstrated to play an important role in Th2-mediated diseases, such as asthma [72]. TSLP is a cytokine that is capable of influencing the maturation of myeloid (CD11c+) dendritic cells upon binding a heterodimeric receptor, formed by the dimerization of the TSLP-receptor and IL-17 receptor alpha. Finally, GMCSF is another cytokine with a demonstrated role in modulating lung inflammation and influencing models of airway hyperreactivity [73]. These are the most prominent epithelial-derived factors that have a role in programming immunity; however, they are not the only ones. They do demonstrate that epithelial-specific signaling can impact the maturation and differentiation of immune cells and ultimately impact a tissue's immunity.

1.9 Ovalbumin as a model of allergic airway inflammation

Previous work in our lab used control and lung, epithelial-specific HIF1α-deficient mice and treated them with cobalt, a metal that elicits an allergic response and promotes HIF signaling. In these experiments, control mice show the typical signs of metal induced injury which include fibrosis and neutrophil recruitment (Th1 mediated) while HIF1α-deficient mice showed a phenotype that resembled asthma, including Th2 cytokines; eosinophil recruitment, mucus cell metaplasia and expression of chitinase-like proteins [74, 75]. These studies showed that in response to the cobalt, there was an apparent switch from a Th1-mediated response to a Th2-mediated response. It was

this observation which led to an interest in determining what would happen if the mice were already experiencing a Th2-mediated response. Would they be biased toward an exacerbated response to that Th2-mediated inflammation? A known inducer of Th2-mediated inflammation is ovalbumin (OVA), leading to the development of an OVA protocol for these epithelial, lung-specific HIF1/2α mice.

Over time, many different models of asthma have been used in the laboratory setting. The very first mouse models were focused on the symptom of bronchoconstriction. This simply means that a smooth muscle agonist, like methacholine, was used to induce bronchoconstriction [76]. Over time, it was determined that inflammation is also a key component to asthma and it was necessary to create a model which also showed this response. Researchers called these models 'allergic asthma', which were established by first sensitizing the animal to a foreign protein, commonly ovalbumin (OVA). The sensitization consists of an intraperitoneal injection of the OVA, with an adjuvant (e.g. aluminum hydroxide). The adjuvant has been shown to strengthen the immune response to the foreign protein [77]. After several days, the immune system will have begun to mount a reaction to the foreign protein. Another sensitization typically follows to enhance the immune response. These two sensitizations are followed by another dose of OVA, but in this case it is administered via inhalation, this is termed the OVA 'challenge'. This model of allergic asthma results in eosinophil recruitment and histological changes in the lung which are typical of asthma [76, 78].

1.10 Hyperoxia and premature births

With increasing technology, the World Health Organization reports that the survival of infants born prematurely is increasing. In the United States, 12-13% of all births are considered 'premature', and the leading cause of death among these premature births is respiratory distress syndrome [79]. A common therapy given to premature infants is supplemental oxygen in an attempt to counteract their underdeveloped lungs. Oxygen levels *in utero* are typically at a pO_2 between 20-30 mmHg, which are the levels the premature infant should be experiencing. However, the alveoli of that premature infant after birth are extremely hyperoxic in ambient air alone (pO_2 = 100 mmHg), and especially with oxygen therapy where the pO_2 can be significantly higher than 100 mmHg. It is known that these hyperoxic conditions can result in several medical issues, such as bronchopulmonary dysplasia, retinopathy of prematurity and changes in innate immunity [80, 81]. Interestingly, premature infants are at an increased risk for atopic diseases, such as asthma and this risk increases with supplemental oxygen therapy. It has been hypothesized that these pathologies are due, at least in part, to the disruption of normal hypoxia-mediated signaling that would occur in utero.

Low levels of oxygen *in utero* are important for normal fetal development, as these hypoxic signals promote branching morphogenesis and bud formation [82, 83]. The levels of oxygen the fetus experiences in utero are very different than the oxygen levels an infant experiences upon birth. Premature infants experience this drastic change in oxygen prior to the lungs being structurally ready. The need for delivery of

supplemental oxygen in these premature infants increases the stress on the underdeveloped tissue and results in abnormal lung development. There have also been studies that have linked premature birth to asthma-like symptoms [84]. Despite these largely epidemiological studies, the understanding of the mechanism linking premature birth and pulmonary dysfunction later in life is not completely understood.

The HIFα mouse model mentioned above removes specific HIFs (either HIF1α, HIF2α, or both HIF1α and HIF2α) from the ATII and Clara cells after dosing with doxycycline (DOX). Given the observed Th2 bias in the HIF1-deficient mice that phenotypically resembles asthma, and that increased oxygen availability can inhibit normal HIF-mediated signaling, it is hypothesized that premature birth-induced lung hyperoxia increases the susceptibility to atopic diseases because of its ability to inhibit epithelial-derived HIF signaling.

1.11 Hypothesis and Specific Aims

The overall hypothesis is that: Epithelial-derived HIF signaling predisposes the lung to an asthma-like pathology following toxicant challenge via cytokine production and lymphocyte recruitment and differentiation. This general hypothesis was tested by the following specific aims:

Aim 1: Characterize the roles of HIF1α and HIF2α in allergic airway inflammation (AAI) response using an ovalbumin (OVA) sensitization and challenge model. Preliminary data suggest that loss of HIFα in the alveolar type II (ATII) and Clara cells of the lung predisposes the lung to an asthma-like phenotype following cobalt exposure [85]. This finding suggests that the HIF-deficient mice should be more susceptible to known models of allergen-induced asthma, such as OVA. To test this hypothesis HIF1α-deficient, HIF2α-deficient and HIF1/2α-deficient mice will undergo the OVA protocol, and functional and inflammatory responses will be determined.

Aim 2: Identify differences within the lung in control and HIF1α-deficient mice that occur during OVA hypersensitivity and determine the role of inflammatory cells and their cytokines, recruitment, and maturation. Preliminary data obtained from characterizing the OVA model for Specific Aim 1 have shown that the loss of HIF1α results in a hypersensitivity to a known Th2-mediated inflammatory response. To determine if this change in inflammatory response is due to the composition of immune cells within the lung, cytokines and inflammatory cells present in control and HIF1α-deficient mice will be characterized.

Aim 3: Investigate if HIF1α stimulation can inhibit the Th2 mediated inflammatory response induced by OVA. The preliminary data obtained from Specific Aim 1 indicate that loss of HIF1α signaling results in an exacerbated response to OVA-induced inflammation. To test whether stimulation of HIF1a might protect against allergen-induced inflammation, the α-ketoglutarate analog, dimethyloxalylyglycine (DMOG), will be used to stimulate HIF signaling, as well as a new transgenic mouse with an inducible, constitutively active form of HIF1α.

CHAPTER TWO

Materials and Methods

2.1 Description of mice

Matings between HIF1$\alpha^{flox/flox}$ (gift from Randall Johnson, University of California–San Diego) and SP-C-rtTA$^{-/tg}$/(tetO)$_7$-CMV-Cre$^{tg/tg}$ (gift from Jeffrey A. Whitsett, Cincinnati Children's Hospital Medical Center) transgenic mice generated the HIF1α triple transgenic mice used in these experiments. These mice, SP-C-rtTA$^{-/tg}$/(tetO)$_7$-CMV-Cre$^{tg/tg}$/HIF1$\alpha^{flox/flox}$, upon exposure to doxycycline (DOX), are able to undergo recombination in the floxed HIF1α gene. The timing of DOX exposure influences the specificty of recombination. If DOX is delivered in utero, recombination is induced in all epithelial cells. If induced post-natally, only ATII and Clara cells of the respiratory epithelium are recombined [75, 86] (Figure 3). The HIF2α (gift from Celeste Simon, University of Pennsylvania) transgenic mice used in the experiments were created in the same way and the HIF1/2α model was generated by mating the HIF1α and HIF2α triple transgenics. Previous studies have demonstrated that the Th2 bias is dependent upon all three transgenes and doxcycline exposure, thus eliminating the possibility that these observed inflammatory changes are due to doxycycline alone or Cre toxicity [75]. All of the mice used in this study are maintained in a mixed C57BL/6 and FVB/N background. Genotyping of the mice was performed by PCR for the three loci as previously described [87].

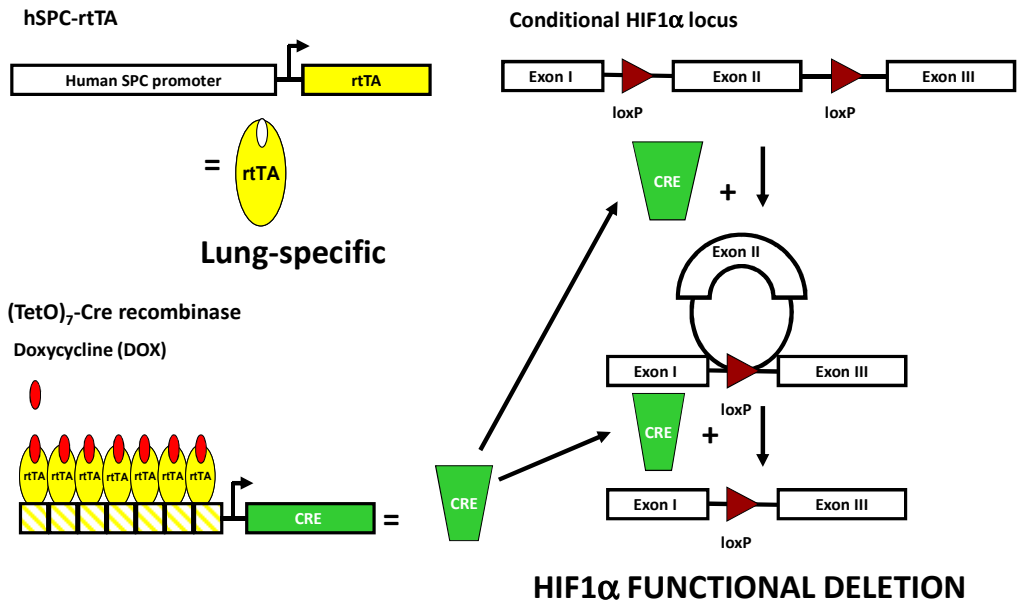


Figure 3 **SPC driven transgenic mouse model**. Transgenic mice were generated that express three transgenes the rtTA protein under the control of the human *SP-C* promoter, the (tetO)7-CMV-Cre recombinase transgene in which Cre recombinase expression is controlled by the (tetO)7-CMV promoter, and the conditional HIF1α locus. In the presence of doxycycline and rtTA, Cre recombinase is induced in a lung-specific manner and facilitates the homologous recombination of the loxP sites flanking exon 2 thus inactivating the HIF1α locus. Similar constructs were used for the HIF2α and HIF1/2α-deficient animals.

A second transgenic mouse model was used for a subset of experiments. The same HIF-1$\alpha^{fl/fl}$ mice mentioned previously were a gift from Randall Johnson (University of California, San Diego), and UBC-Cre-ERT2$^{+/-}$ mice were obtained from Jackson Laboratories (Bar Harbor, ME). Expression of the Cre-ERT2 transgene is regulated by the ubiquitin C (UBC) promoter and is expressed in all cell types. Cre-ERT2 is a fusion protein composed of Cre recombinase and a mutated estrogen receptor that is selectively activated and targeted to the nucleus when in the presence of (Z)-1-(p-Dimethylaminoethoxyphenyl)-1,2-diphenyl-1-butene, *trans*-2-[4-(1,2-Diphenyl-1-butenyl)phenoxy]-*N*,*N*-dimethylethylamine [Tamoxifen (TAM)], but not

estrogen [88]. C57Bl/6 HIF-1α$^{fl/fl}$ and UBC-Cre-ERT2$^{+/-}$ transgenic mice were mated to generate UBC-Cre-ERT2$^{+/-}$/HIF-1α$^{fl/fl}$ mice which can be induced to recombine the floxed HIF-1α gene when treated with TAM (Figure 4) [89]. Female UBC-Cre-ERT2$^{+/-}$/HIF-1α$^{fl/fl}$ mice (4-5 weeks old) were treated once per day for 5 days with 200 µg/g body weight TAM in corn oil or with only corn oil as vehicle control (CTRL) by oral gavage [88]. TAM-treated UBC-Cre-ERT2$^{+/-}$/HIF-1α$^{fl/fl}$ mice were HIF-1α deficient and CTRL-treated animals were HIF-1α sufficient.

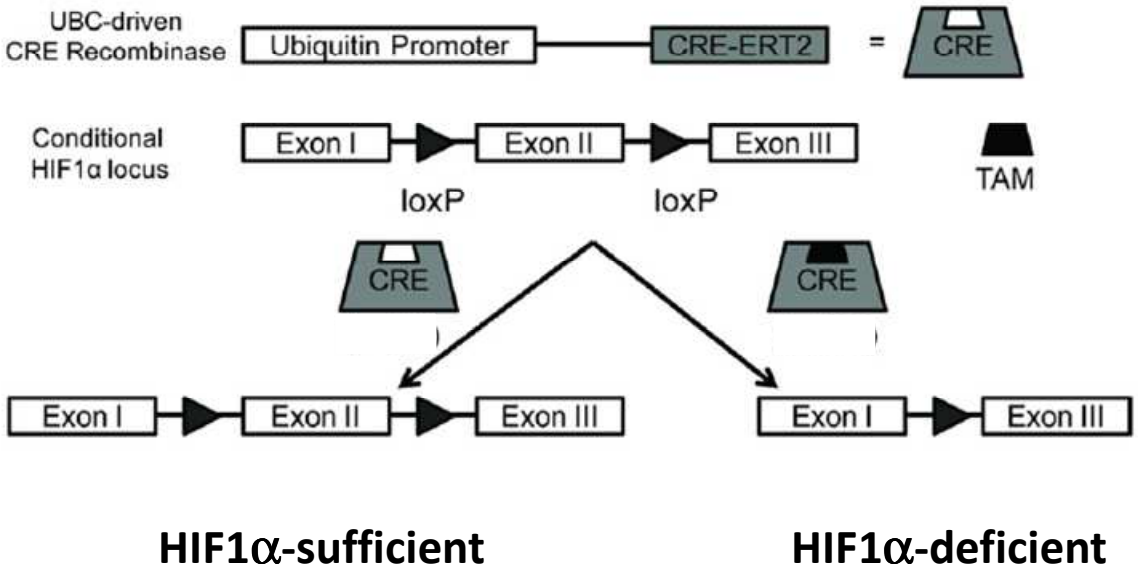


Figure 4 **UBC driven transgenic mouse model**. Four to five week old female CRE-ERT2$^{(+/-)}$ /HIF-1α$^{fl/fl}$ mice were treated with corn oil (CTRL) or 200 µg/g TAM for five days to generate mice HIF-1α-deficient in all cell types.

2.2 Doxycycline treatment and animal husbandry

Exposing triple transgenic mice to DOX *in utero* is lethal upon birth [87]. Postnatal exposure leads to almost complete loss of HIF1α from Clara and ATII cells. In the developmental model, lactating dams were exposed to DOX-containing feed (625 mg doxycycline/kg; Harlan Teklad, Madison, WI) and drinking water (0.8 mg/ml, MP Biochemicals, Solon, OH) beginning on postnatal day 4 (PN4) until weaning. This dose of doxycycline is slightly lower than the concentration that has been used to induce recombination without any observable toxicity or impact on alveolarization [90]. The triple transgenic mice were maintained on the same DOX-containing food and water until they were approximately 4 weeks of age (PN28). DOX treatment was terminated approximately 2 weeks before the first intraperitoneal (i.p.) injection of OVA (Figure 5). These mice will be referred to as PN4 mice throughout the paper. In the young adult model, mice were treated with DOX for 10 days, from PN32 to PN42. DOX was again terminated approximately 2 weeks before the first i.p. injection of OVA (Figure 5). These mice will be referred to as PN32 mice throughout the paper. The third DOX treatment regimen began at PN4, but only went until PN14. This was to match the shortened length of DOX exposure in the PN32 mice. These mice will be referred to as PN4-14. The OVA sensitizations for this group began at the same age as those in the PN4 group described previously. All DOX treated mice will be referred to as HIF1α-deficient throughout this paper. Animals used as controls in this study were also triple transgenic [SP-C-rtTA$^{-/tg}$/(tetO)$_7$-CMV-Cre$^{tg/tg}$/HIF1α$^{flox/flox}$] mice which were given normal food and water ad libitum. All the procedures regarding the handling, maintenance, and necropsy protocols of the mice used in this study were approved by

the university laboratory animal resource (ULAR) regulatory unit at Michigan State University.

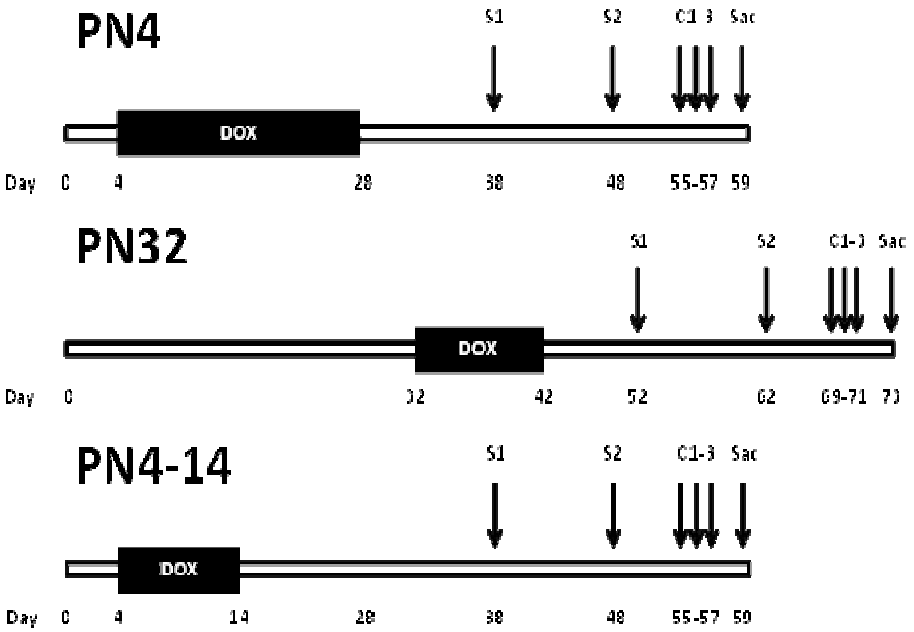


Figure 5 **Experimental design**. Hypoxia inducible factor-1α (HIF-1α)-deficient mice were generated through 3 different postnatal doxycycline (DOX) treatment schemes: postnatal day (PN)4, PN32, or PN4–14. For PN4, DOX was delivered from PN4 to PN28. For PN32, DOX was delivered from PN32 to PN42. For PN4–14, DOX was delivered from PN4 to PN14. Following at least 10 days on normal food and water, HIF-1α-sufficient and HIF-1α-deficient female mice were sensitized (S1) intraperitoneally with saline or ovalbumin (OVA) plus alum (250 μl) and 10 days later sensitized again (S2) with saline or OVA (no alum, 250 μl). Seven days later, the mice were challenged (C1–3) with saline or OVA (1% solution) via inhalation 30 min per day for 3 days. Animals were euthanized 48 h after their final challenge.

2.3 Ovalbumin exposure, tissue harvesting, and processing

To determine the effect of the lung-specific, epithelial loss of HIF1α in the OVA model of allergic airway disease, control and HIF1α-deficient female mice were randomly assigned to saline or OVA treatment groups. Approximately 2 weeks after

terminating DOX exposure, mice were injected with OVA/alum (20 μg/1mg, i.p. in 250μl saline). Saline group animals were injected with 250 μl saline alone. Ten days later, OVA group animals were given an OVA boost consisting of an injection of OVA alone (20 μg, i.p. in 250μl saline) and 250 μl saline for the saline group. One week after the OVA boost, mice were challenged with either OVA (OVA group) or saline (saline group) via inhalation (Figure.5). Briefly, mice were exposed to OVA (1% in saline) or saline alone in a mass dosing chamber (Buxco, Wilmington, NC) with a nebulizer (Aerogen, Galway, Ireland) for 30 minutes at 100% duty and a flow rate of 2.5 l/min, once daily, for three consecutive days. Mice were anesthetized with pentobarbital sodium (50 mg/kg) 48 hours after final OVA/saline challenge, and a midline laparotomy was performed. Tissue isolation and bronchoalveolar lavage fluid (BALF) were collected as described previously [74]. Total and differential cell counts were performed on BALF samples using a hemacytometer and Diff-Quik reagent (Baxter, Deerfield, IL), respectively. The right lung lobes were removed and snap frozen in liquid nitrogen. The left lung lobe was perfused with 10% neutral buffered formalin for 1 hour at 30 cm pressure and then stored for at least 24 hours in a large volume of fixative before processing for histopathological analysis. After fixation, the left lobe was microdissected along the main axial airway, and two transverse tissue blocks were excised at the level of the fifth (proximal) and eleventh (distal) airway generation (G5 and G11, respectively) for further processing, as has been previously reported [91].

2.4 Dimethyloxyalylglycine (DMOG) treatment of mice

In select experiments, female mice (HIF1$\alpha^{fl/fl}$) were treated with the prolyl hydroxylase domain containing protein (PHD) inhibitor, dimethyloxyalylglycine (DMOG) to determine if activation of HIF-mediated signaling miught protect agains allergen-induced inflammation.. There was no dosing of DOX, so although the mice were HIF1$\alpha^{fl/fl}$, none of the mice were HIF1α-deficient. Individual groups received either SAL or 1% OVA solution via inhalation for three days after the appropriate sensitization protocol (Figure 5, refer to PN4 protocol, but disregard DOX treatment). A subset of each of these groups (i.e. SAL and OVA) was also given DMOG (300 mg/kg, i.p.) on days 54 and 56 [92]. Mice were euthanized 48 hours after last exposure and were processed as described previously with collection of BALF, among other samples.

2.5 Hyperoxia treatment of mice

Mice, SP-C-rtTA$^{-/tg}$/(tetO)$_7$-CMV-Cre$^{tg/tg}$/HIF1$\alpha^{flox/flox}$, were also used in experiments to determine the potential phenotypic overlap between hyperoxia and loss of epithelial-derived HIF signaling. As in the DMOG experiments, no DOX was provided, therefore, all mice are genotype matched to the HIF1a-deficient experiments, but are HIF1α-sufficient. Mice were exposed to room air (RA) or hyperoxia (75% O_2) immediately following birth (PN0) until PN14. Mice were maintained at 75% O_2 in an oxygen workstation (Coy Laboratories, Grass Lake, MI) and nursing mothers were rotated every 12 hours. These mice were then sensitized and challenged with OVA

according to the OVA treatment protocol (Figure 5, refer to PN4 protocol, but disregard DOX treatment) upon reaching early adulthood.

2.6 Histopathology and immunohistochemistry

All samples from each treatment group were analyzed for histopathological changes as previously described [74]. Briefly, fixed left lung lobe tissues were embedded in paraffin, and sections (5 μm) were mounted on glass slides and stained with hematoxylin and eosin (H&E), Alcian Blue (pH 2.5)/Periodic Acid-Schiff (AB/PAS) to detect total mucosubstances (acidic and neutral) in airway epithelium, or immunostained for major basic protein (MBP) within eosinophils using a polyclonal rabbit antibody directed against murine MBP (1:500, Mayo Clinic, Scottsdale, AZ), HIF1α antibody, HIF2α antibody (both 1:100, Novus Biologicals, Littleton, CO), Galectin-3 antibody (1:500, kind gift of Dr. John L. Wang, Michigan State University), or IL-1R2 antibody (1:10, R&D Systems, Minneapolis, MN).

2.7 Morphometry of intraepithelial mucosubstances

To determine the amount of stored mucosubstances in the epithelium lining, the proximal axial airway (generation 5) in the left lung lobe volume densities of AB/PAS stained samples were analyzed using computerized image analysis. The area of positive AB/PAS staining was calculated using the Scion Image program (Scion Corporation, Frederick, MD). The length of the basal lamina underlying the airway surface epithelium was calculated from the contour length of the digitized image of the basal lamina. The volume of stored mucosubstances (volume density [Vs]) per unit of surface area was estimated using the method previously described in detail by Harkema et al [93].

2.8 Pulmonary function analysis

Mice were anesthetized with pentobarbital sodium (120 mg/kg i.p.),intubated and ventilated with a small animal ventilator (SAV, *flexiVent*; SCIREQ, Montreal, Quebec, Canada) at an initial frequency of 120-150 breaths/minute and volume of 1.5 ml/kg. Increasing doses of aerosolized methacholine were given via an Aeroneb nebulizer (Aerogen, Galway, Ireland) and total lung resistance was calculated during ventilator-controlled oscillations in breathing (sinusoidal forced oscillations) as determined by protocols specific to the f*lexiVent* pulmonary function testing system (SnapShot-150, SCIREQ). Data collection took approximately 10 minutes, after which animals were euthanized and tissues collected as described above.

2.9 Cytokine bead array

BALF was analyzed for cytokine expression (IL-2, IL-4, IL-5, INFγ, TNFα, IL-13, KC and IL1β) using Cytometric Bead Array kits and reagents (BD Bioscience, Franklin Lakes, NJ) according to manufacturer's protocols. In brief, cytokine-specific antibody coated beads are mixed with the BALF samples and then run through a flow cytometer for analysis based on the specific fluorescent signature of each bead. Cytokine concentrations were calculated using standard curves generated from samples supplied by the manufacturer.

2.10 Flow cytometry on lung cells

Lung tissue was homogenized by manual dissociation using a cell strainer (Sigma), and cells were placed in RPMI medium with 2% fetal bovine serum (FBS). To analyze for cytokine production, cells were restimulated in vitro with phorbol 12-myristate 13-acetate (PMA, 40 nM) and ionomycin (Io, 0.5 μM) (Sigma) for 4-6 hours. For intracellular cytokine staining, the PMA/Io incubation was performed in the presence of Brefeldin A (BioLegend, San Diego, CA, USA) via manufacturer's instructions. These cells were then washed three times with fluorescence-activated cell sorting (FACS) buffer (1x Hank's Balanced Salt Solution (HBSS), 1% Bovine serum albumin (BSA), 0.1% sodium azide, pH = 7.6), and Fc receptors were blocked using purified rat anti-mouse CD16/32 (BioLegend, San Diego, CA, USA). Fc receptors are proteins found on the surface of some cells, including dendritic cells, that aid in the protective nature of the immune system. These Fc receptors were blocked to limit non-specific binding of the antibodies. Surface markers were stained with specific antibodies from BioLegend: CD11b, CD11c, Gr-1, CD317, B220, MHC II, F4/80, CD86. Cells were fixed with Cytofix (Becton Dickinson, Franklin Lakes, NJ, USA) before flow cytometric analysis. All samples were prepared in 96-well round-bottom plates and analyzed using a BD Biosciences FACSCanto II flow cytometer (San Jose, CA, USA). Data were analyzed individually and concatenated for graphical representation using Kaluza v1.1.

2.11 Quantitative analysis

Treatment and HIF1α-dependent differences in BALF cells and cytokines, intraepithelial mucosubstances, and airway resistance were analyzed by one-way ANOVA followed by Fishers test (OriginPro, OriginLab, Northampton, MA). All error

bars represent standard error of the mean (SEM). Statistical differences of *P* value < 0.05 were considered significant.

CHAPTER THREE

Results

Including:

Greenwood, K.K., Proper, S.P., Saini, Y., Bramble, L.A., Jackson-Humbles, D.N., Wagner, J.G., Harkema, J.R., and LaPres, J.J. (2012). Neonatal epithelial hypoxia inducible factor-1α expression regulates the response of the lung to experimental asthma. Am J Physiol Lung Cell Mol Physiol. 302: L455-L462.

3.1 HIF1/2α Immunohistochemistry

To verify the loss of HIF1α and HIF2α in the different mouse models, immunohistochemistry was performed on lung samples. Loss of HIF1α in the HIF1α$^{fl/fl}$ mice was shown in a previous lab publication by Saini et al. [74] (Figure 6). Following a similar DOX administration in food and water protocol, dosing for approximately 4

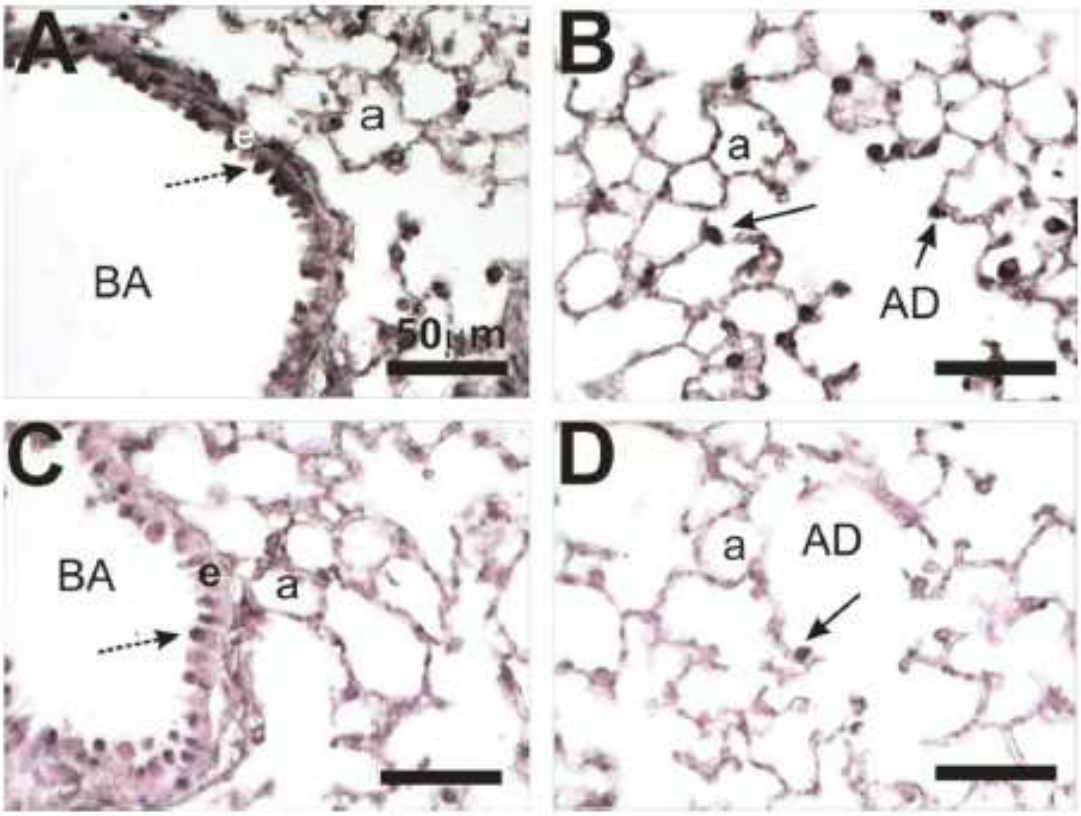


Figure 6 **Hypoxia-inducible factor 1α (HIF1α) immunohistochemistry of lungs from control and doxycycline-treated mice**. Lung tissue sections from control (**A and B**) and mice that were HIF1α-deficient in their lungs (**C and D**) were analyzed by immunohistochemistry using a HIF1α-specific antibody. Control (**A and B**) and doxycycline (**C and D**)-treated mice were compared. HIF1α staining is prominent in the epithelial cell (**e**) lining the bronchiolar airway (**BA**) (**dashed arrow**) and type II cells (**solid arrow**) in the alveolar duct (**AD**) and alveolus (**a**). Staining is greatly reduced in the doxycycline-treated animals (**C and D**).

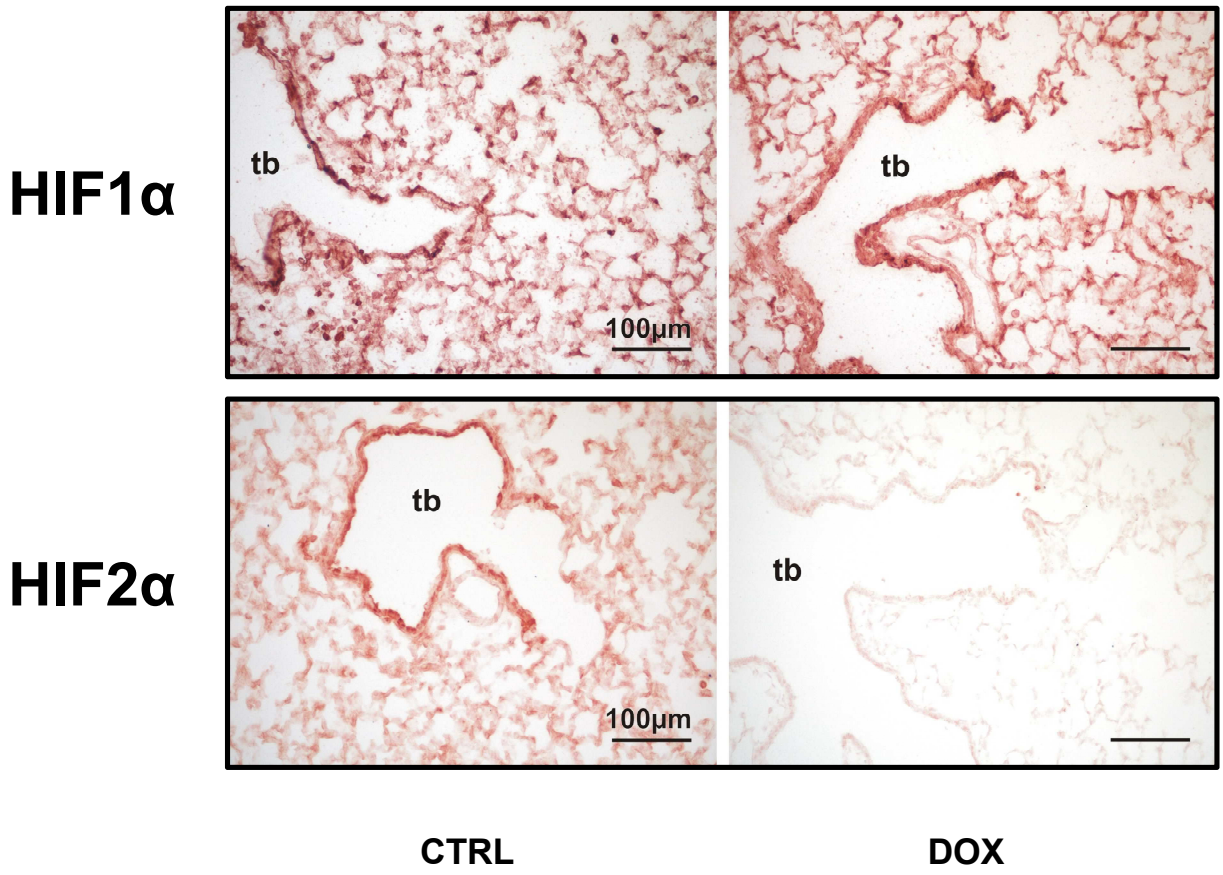


Figure 7 **HIF1α and HIF2α Immunohistochemistry in HIF2α$^{fl/fl}$ mice**. Both HIF1α (**top left and right**) and HIF2α (**bottom left and right**) expression was assessed using immunohistochemistry in the HIF2α$^{fl/fl}$ mice. HIF2α$^{fl/fl}$ mice fed regular food and water (**CTRL, left top and bottom**) or fed food and water containing DOX (**DOX, right top and bottom**). There is no loss in HIF1α staining, while there is a noticeable absence of HIF2α in the DOX treated mice. No counterstain was used to highlight the retention of HIF1α expression and the loss of HIF2α expression.

weeks (PN4-PN28) was sufficient to significantly reduce HIF2α levels in the HIF2α$^{fl/fl}$ mice treated with DOX (Figure 7). In contrast, HIF1α staining remained consistent in both regular and DOX treated HIF2α$^{fl/fl}$ animals. Administration of DOX for approximately 4 weeks (PN4-PN28) was also sufficient to induce recombination and

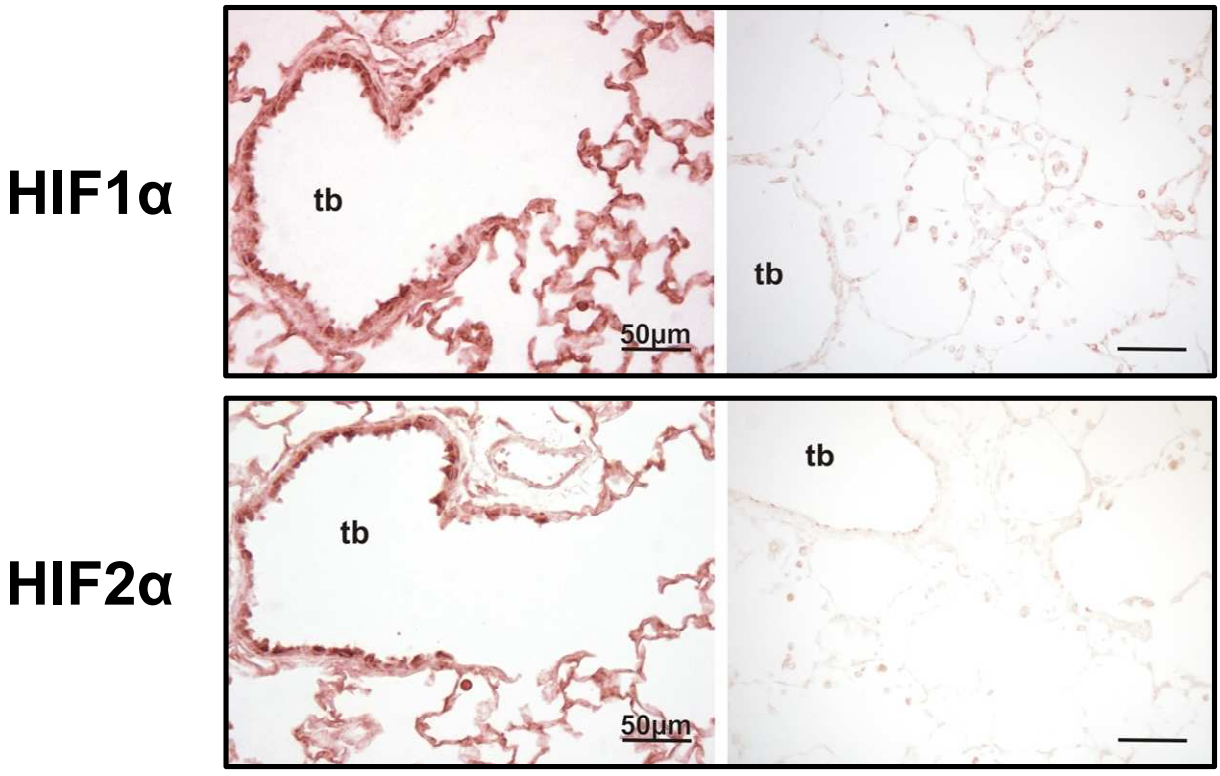


Figure 8 **HIF1α and HIF2α Immunohistochemistry in the HIF1/2α$^{fl/fl}$ mice**. Both HIF1α (**top left and right**) and HIF2α (**bottom left and right**) expression was assessed using immunohistochemistry in the HIF1/2α$^{fl/fl}$ mice. HIF1/2α$^{fl/fl}$ fed regular food and water (**CTRL, left top and bottom**) or fed food and water containing DOX (**right top and bottom**). There is a loss in both HIF1α and HIF2α in the DOX-treated mice. No counterstain was used to highlight the loss of both HIF1α and HIF2α expression.

significantly reduce both HIF1α and HIF2α levels in HIF1/2α$^{fl/fl}$ mice (Figure 8). HIF1α and HIF2α staining is present in the control group (No DOX, HIF1/2α-sufficient), and was significantly decreased in the DOX group (HIF1/2α-deficient). No counterstain was used to help visualize the differential HIF1α and HIF2α staining.

3.2 Cell counts from lavage fluid of HIF1α-sufficient and PN4 HIF1α-deficient animals.

Total cells recovered from BALF increased slightly with OVA treatment in HIF1α-sufficient control mice, and this rise in total cells was exacerbated in the PN4 HIF1α-deficient mice treated with OVA. Differential staining of the cells recovered in the BALF showed that MBP-containing eosinophils were the predominant cell type causing the increase in total BALF inflammatory cells in both the OVA-treated control and PN4 HIF1α-deficient groups. In both HIF1α-sufficient and HIF1α-deficient saline-treated groups, eosinophils accounted for approximately 0-4% of the total cells. In comparison, with OVA treatment of HIF1α-sufficient animals, this number rose to 33%, but with OVA treatment in PN4 HIF1α-deficient animals it almost doubled, reaching 59%. In addition, macrophages were also significantly increased in the OVA-treated PN4 HIF1α-deficient mice compared with both the saline-treated deficient mice and the OVA-treated HIF1α-sufficient mice (Figure 9). Despite this increase, the overall percentage of macrophages was actually reduced from 92-97% of total cells in saline-treated mice to 62% and 38% of total cells in HIF1α-sufficient and HIF1α-deficient OVA-treated mice, respectively. The number of lymphocytes was also significantly increased in the OVA-treated PN4 HIF1α-deficient mice compared with saline-treated, deficient mice, however, the percentage of lymphocytes in the total number of cells remained relatively steady in these two groups at 2.6% and 1.4%, respectively.

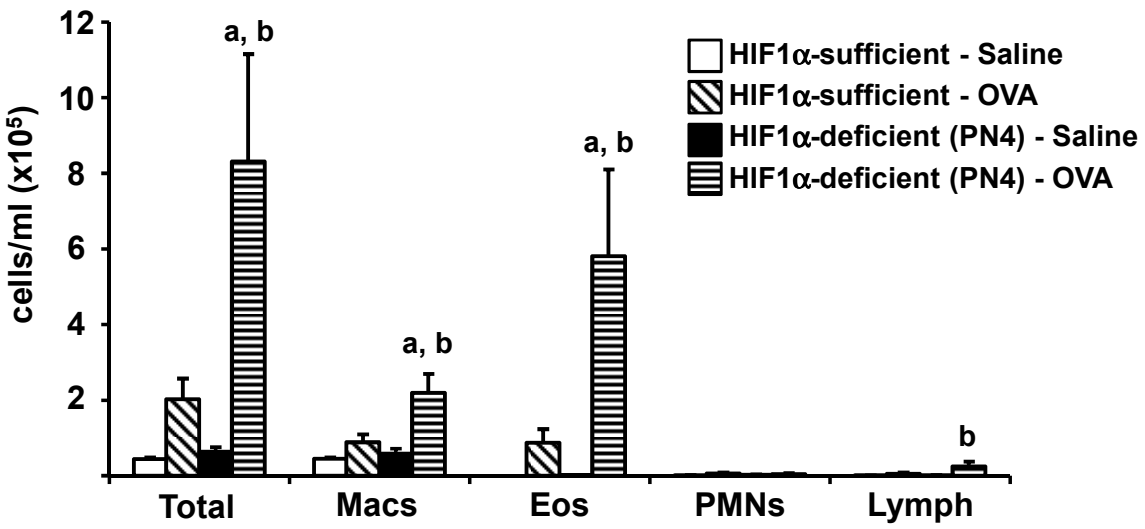


Figure 9 **The effect of OVA on BALF cellularity (PN4 mice)**. HIF1α-sufficient (**white and diagonally hatched bars**) and PN4 HIF1α-deficient (**black and horizontally hatched**) mice were sensitized/challenged with saline (white and black bars) or OVA (**diagonally and horizontally hatched bars**). Total cells, as well as the numbers of macrophages (Macs), eosinophils (Eos), neutrophils (PMNs), and lymphocytes (Lymph) were determined from BALF. Values are the mean +/- SEM (n ≥ 9). a = significantly different from HIF1α-sufficient within treatment $p < 0.05$, b = significantly different from saline treated within genotype $p < 0.05$.

3.3 Bronchoalveolar lavage fluid (BALF) cellularity of HIF2α-sufficient and HIF2α-deficient animals treated with OVA

Total cells recovered from BALF increased with OVA treatment in both HIF2α-sufficient and HIF2α-deficient animals. There was no significant difference in total cells when comparing the HIF2α-sufficient and HIF2α-deficient animals. Differential staining was performed on the cells recovered in the BALF to determine if loss of HIF2α altered the response to OVA at the level of infiltrate. These cell counts showed that the majority of the OVA-induced increase in total cells was due to eosinophils in both the

HIF2α-sufficient and HIF2α-deficient mice. However, there was no significant difference between these two groups for any cell type (Figure 10). In mice exposed to saline, eosinophils accounted for 0-1.2% of total cells. Following OVA treatment, in HIF2α-sufficient animals, the eosinophils rose to 68.1% of total cells, while similarly treated HIF2α-deficient animals had eosinophil increases to 55.4%. Both groups (i.e. HIF2α-sufficient and deficient) displayed a statistically significant increase in eosinophils upon OVA treatment when compared to the saline treated animals, however, the HIF2α-sufficient and HIF2α-deficient animals were not different in their response to OVA (Figure 10). Additionally, macrophages were also significantly increased in both HIF2α-sufficient and HIF2α-deficient OVA treated animals when compared to their saline treated controls. Even though the total count of macrophages was significantly increased upon OVA treatment, the percentage of total cells that were macrophages fell from between 95-98% in saline treated mice to 24-33% in OVA treated mice. The numbers of neutrophils were also significantly increased following OVA treatment, but not significantly different when comparing HIF2α-sufficient to HIF2α-deficient groups. Neutrophils accounted for just 0.3-0.5% of total cells in saline treated and rose significantly in OVA treated animals, however they still only accounted for 2.7% of total cells in HIF2α-sufficient and 3.2% in HIF2α-deficient mice. Lastly, lymphocytes were significantly increased with OVA treatment in the HIF2α-deficient mice when compared to saline treated controls within genotype, increasing from 1.8% of total cells with saline treatment to 8.4% upon OVA treatment.

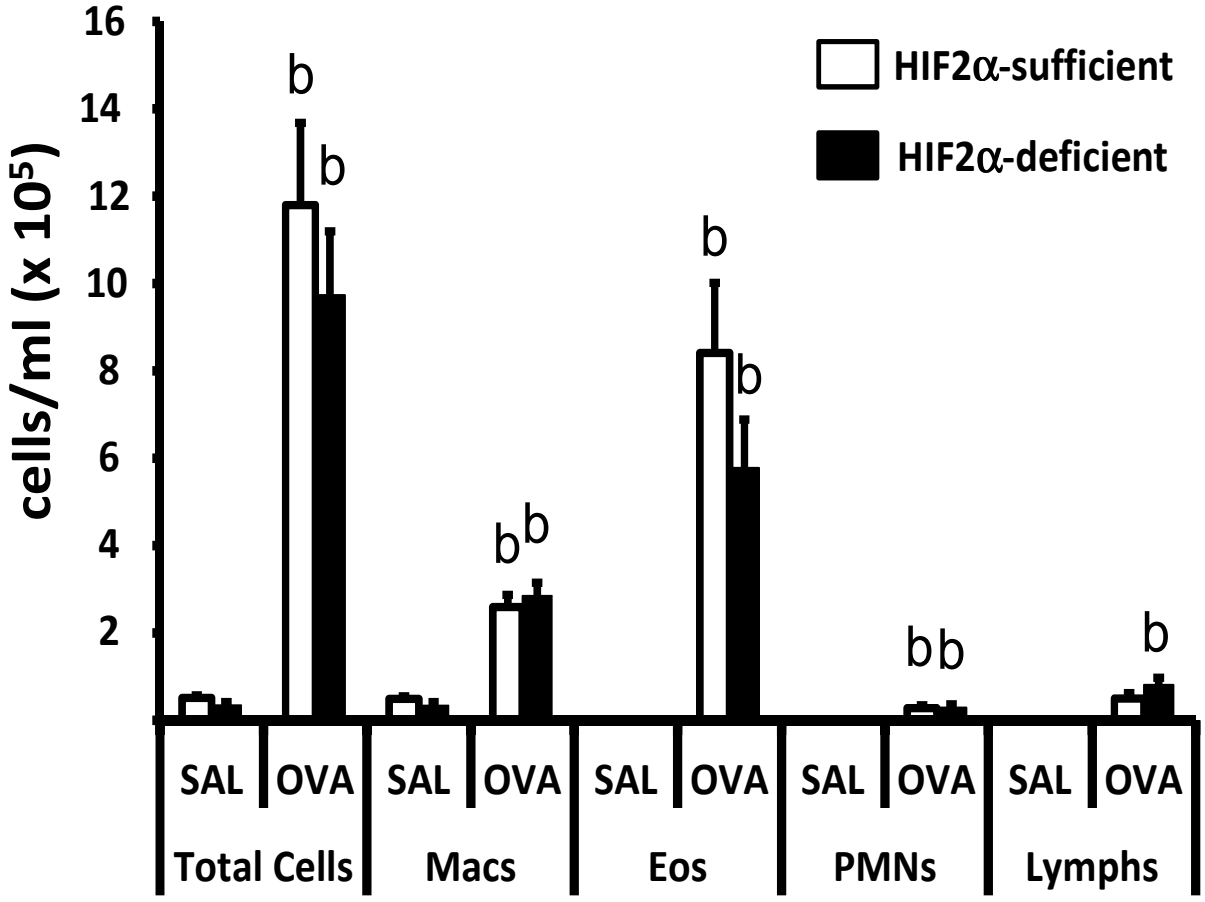


Figure 10 **The effect of OVA on BALF cellularity in HIF2α mice**. HIF2α-sufficient (**white bars**) and PN4 HIF2α-deficient (**black bars**) mice were sensitized/challenged with saline (**SAL**) or ovalbumin (**OVA**). Total cells, as well as the numbers of macrophages (Macs), eosinophils (Eos), neutrophils (PMNs), and lymphocytes (Lymphs) were determined from BALF. Values are the mean +/- SEM ($n \geq 9$). b = significantly different from saline treated within genotype $p < 0.05$, ANOVA.

3.4 BALF cellularity of HIF1/2α-sufficient and HIF1/2α-deficient animals treated with OVA

Total cells were analyzed after recovery in BALF for saline and OVA treated HIF1/2α-sufficient and HIF1/2α-deficient mice. Cell counts were somewhat variable, so

no significant differences were found, however some interesting trends were noted. OVA treatment in HIF1/2α-sufficient mice led to an expected increase in total cells. Interestingly, the HIF1/2α-deficient mice seemed to be partially protected from OVA-induced cellular infiltration in the BALF, as total cell counts were decreased compared to the OVA treated HIF1/2α-sufficient group (Figure 11). After differential staining was performed, it was clear that upon OVA treatment there was a switch from predominantly macrophages (95% in HIF1/2α-sufficient mice and 98% in HIF1/2α-deficient mice) and no eosinophils, to approximately half eosinophils (54% of total cells in HIF1/2α-sufficient

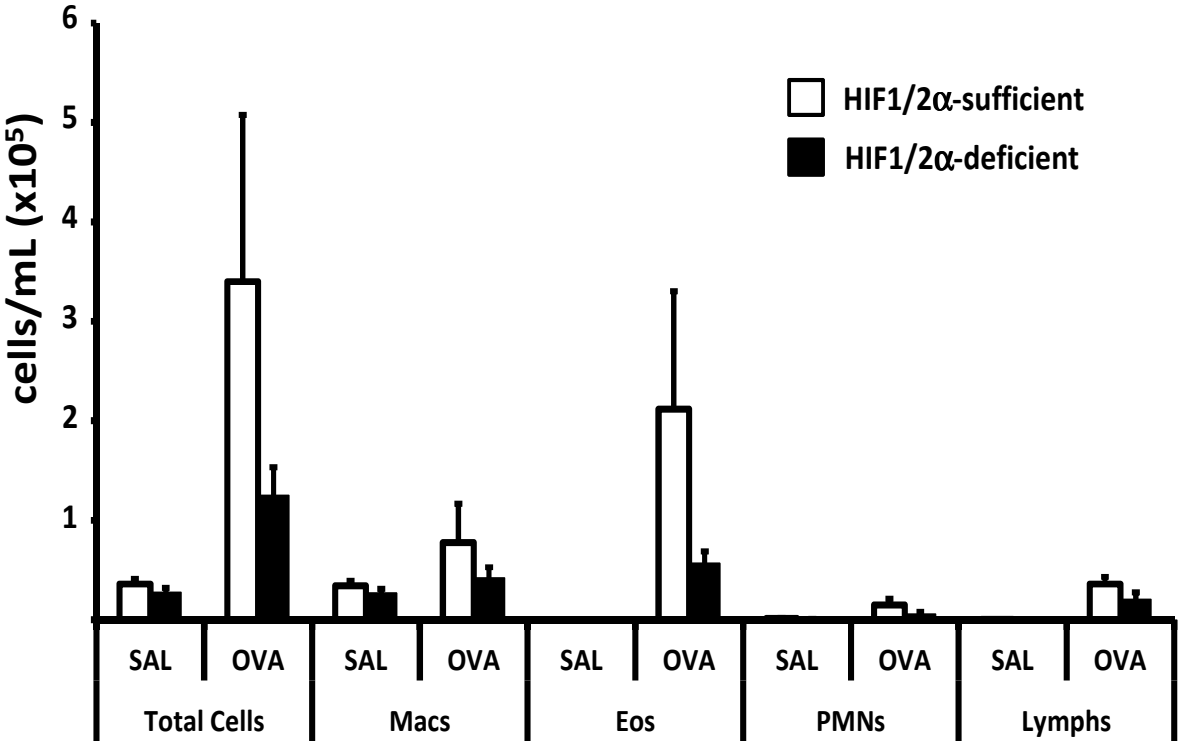


Figure 11 **The effect of OVA on BALF cellularity in HIF1/2α mice**. HIF1/2α-sufficient (**white bars**) and PN4 HIF1/2α-deficient (**black bars**) mice were sensitized/challenged with saline (**SAL**) or ovalbumin (**OVA**). Total cells, as well as the numbers of macrophages (Macs), eosinophils (Eos), neutrophils (PMNs), and lymphocytes (Lymphs) were determined from BALF. Values are the mean +/- SEM.

and 47% in HIF1/2α-deficient mice) and only a quarter macrophages (24% of total cells in HIF1/2α-sufficient and 33% in HIF1/2α-deficient mice). From this differential data, it seems the cellular make-up of the infiltrate is consistent with OVA treatment, independent of the loss of HIF1/2α, but the total cells and eosinophils seems to decrease in the HIF1/2α-deficient mice (Figure 11).

3.5 Quantification of mucus in HIF1α-sufficient and HIF1α-deficient mice

OVA-induced changes to the airway epithelium included epithelial thickening (hypertrophy) and mucous cell metaplasia (MCM; Figure 12A-D). MCM is defined as the presence of AB/PAS-stained mucous goblet cells in airway epithelium that is normally devoid or contains only occasional secretory cells of this phenotype. Epithelial MCM and hypertrophy were not present in the airways of saline-treated HIF1α-sufficient control mice. MCM was a consistent airway epithelial change in the large-diameter proximal axial airways (e.g., G5) in all of the OVA-treated mice. This alteration was less evident in the epithelium lining of the more distal small-diameter bronchioles. The magnitude of this OVA-induced remodeling of the airway epithelium was similar among all the OVA-treated groups, regardless of HIF1α phenotype (Figure 12E). Since there was no difference in mucus quantification between the HIF1α-sufficient and HIF1α-deficient mice, we did not perform this same analysis on the HIF2α$^{fl/fl}$ or HIF1/2α$^{fl/fl}$ mice.

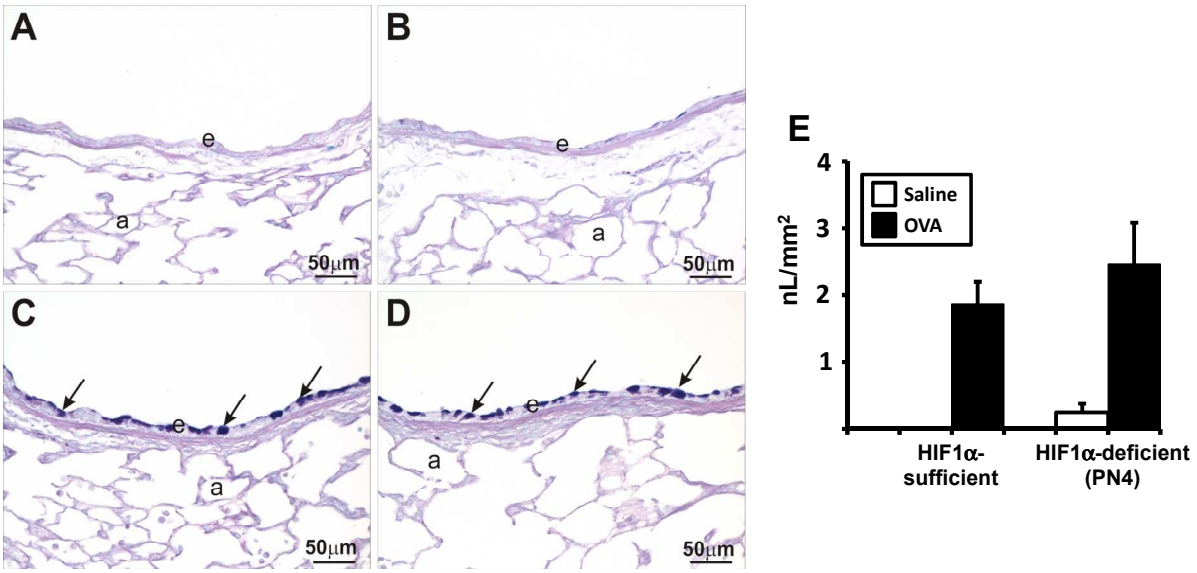


Figure 12 **Alcian Blue–Periodic Acid Schiff (AB-PAS) staining and mucous quantification**. Lung sections from saline (**A, B**) and ovalbumin (**C, D**) sensitized and challenged HIF1α-sufficient (**A, C**) and PN4 HIF1α-deficient (**B, D**) mice were stained with AB-PAS. Solid arrows indicate areas of positive AB-PAS staining (a = alveolus, e = epithelium). Quantification of volume density of mucous (nL/mm^2) from AB-PAS immunohistochemistry from saline (**white bars**) and OVA (**black bars**) control and PN4 HIF1α-deficient mice was performed as described in materials and methods (**E**).

3.6 Histopathology of allergic airways visualized with hematoxylin and eosin (H&E).

In all three floxed HIFα mice (HIF1α, HIF2α and HIF1/2α) only mice sensitized and challenged with OVA had conspicuous histopathological changes in the lung that were consistent with allergic airway disease, visible with hematoxylin and eosin (H&E) staining. These OVA-induced changes consisted of both periairway inflammation and airway epithelial remodeling (i.e., mucous cell metaplasia) and were more prominent in the hilar region of the lung lobe (e.g., G5 lung section) than in the more distal aspects of the lung lobe (i.e., G11 lung section). Both inflammatory and epithelial changes were

also more apparent in the large-diameter, proximal bronchioles (e.g., axial airway) than in the more distal, small-diameter bronchioles (i.e., preterminal and terminal bronchioles). OVA-induced airway inflammation was characterized by a mixed inflammatory cell infiltrate composed of numerous eosinophils, lymphocytes and lesser numbers of neutrophils, along with edema in the interstitium surrounding the airway (peribronchiolar inflammation). OVA-induced inflammatory changes were more prominent in the lungs of PN4 HIF1α-deficient mice compared with similarly treated HIF1α-sufficient mice. Lung sections from saline (Figure 13 A,C) and OVA (Figure 13

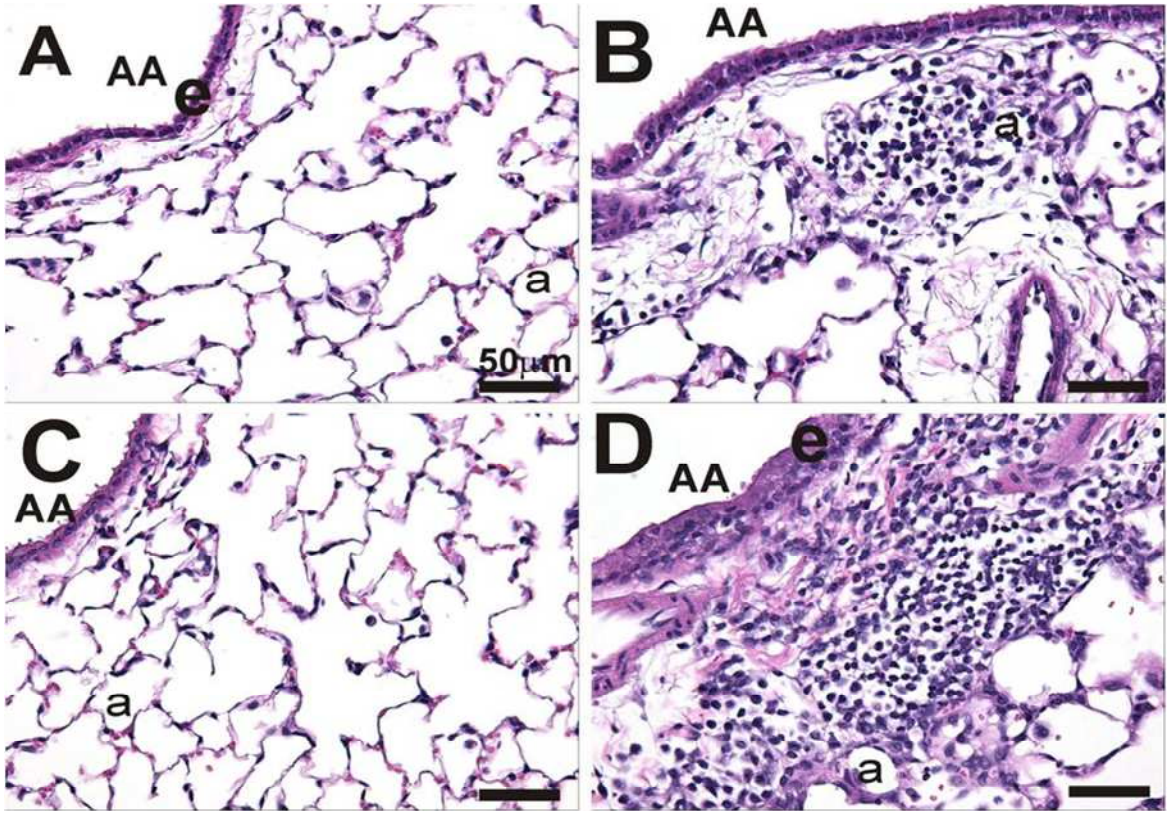


Figure 13 **Histopathology of saline and ovalbumin sensitized and challenged control and HIF1α-deficient mice**. Lung sections from saline (**A and C**) and ovalbumin (**B and D**) sensitized and challenged HIF1α-sufficient (**A and B**) and PN4 HIF1α-deficient (**C and D**) mice. Sections are stained with H&E (a = alveoli, e = epithelium, AA = axial airway).

B,D) sensitized and challenged HIF1α-sufficient (Figure 13 A,B) and PN4 HIF1α-deficient (Figure 13 C,D) lungs were stained with H&E. As mentioned, the excess of cellular infiltrates is only present in the OVA sensitized and challenged lungs with more severe increases in PN4 HIF1α-deficient lungs. The thickening of the airway epithlium is also only visible in OVA sensitized and challenged lungs and is exacerbated in the PN4 HIF1α-deficient samples.

In HIF2$\alpha^{fl/fl}$ mice, only OVA sensitized and challenged mice showed increases in cellular infiltrates and thickening of the airway epithelium, though there were no differences between HIF2α-sufficient and HIF2α-deficient lungs (Data not shown).

In HIF1/2$\alpha^{fl/fl}$ mice, again, only OVA sensitized and challenged mice showed increases in cellular infiltrates and thickening of the airway epithelium, saline treated mice of either genotype did not show this difference. Upon OVA treatment, many cellular infiltrates are observable, most concentrated around the terminal bronchioles of the lungs. The cellular infiltrates are most abundant in HIF1/2α-sufficient mice and decrease with the loss of HIF1α and HIF2α (Figure 14).

3.7 Major Basic Protein (MBP) Immunohistochemistry

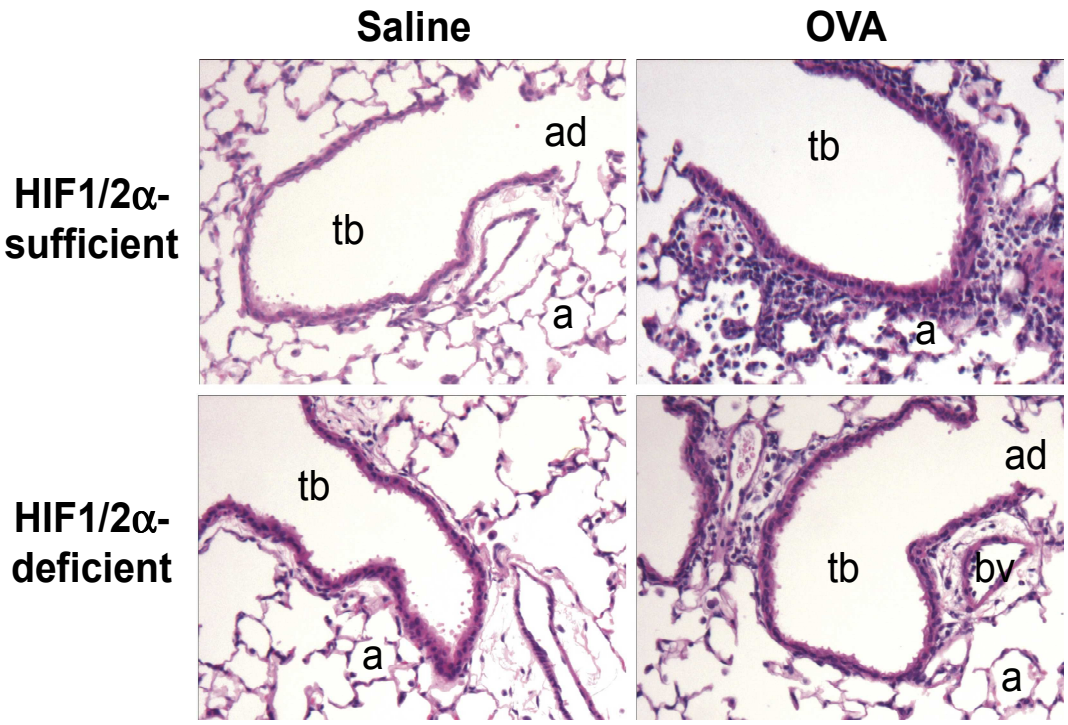


Figure 14 **H&E of HIF1/2α lungs**. Light photomicrographs of a centriacinar region from the lungs of HIF1/2α-sufficient (**top: left and right**) and HIF1/2α deficient (**bottom: left and right**) mice treated with saline (**left: top and bottom**) or OVA (**right: top and bottom**). Tissues were stained with hematoxylin and eosin (a = alveolus, bv = blood vessel, tb = terminal bronchiole, ad = alveolar duct).

Only mice sensitized and challenged with OVA had positive MBP staining, a protein specific to eosinophils. Saline treated mice of either genotype showed no positive MBP staining. This eosinophilic cell infiltrate was localized in the surrounding interstitium, predominantly on the pulmonary artery/arteriole side of the airway, and also circumscribed this airway-associated blood vessel (perivascular inflammation; Figure15). A similar perivascular inflammatory cell infiltrate was also present in the interstitium surrounding some of the pulmonary veins embedded in the alveolar parenchyma located remotely from conducting airways. Interestingly, the targeted

depletion of HIF1α in ATII cells and Clara cells with the PN4 DOX dosing scheme resulted in an extension of the OVA-induced eosinophilic and mononuclear inflammatory response into the proximal alveolar ducts and adjacent alveolar parenchyma deep in the lung (Figure 15) of these mice. PN4 HIF1α deficiency also enhanced the inflammatory response around small diameter airways (i.e., preterminal

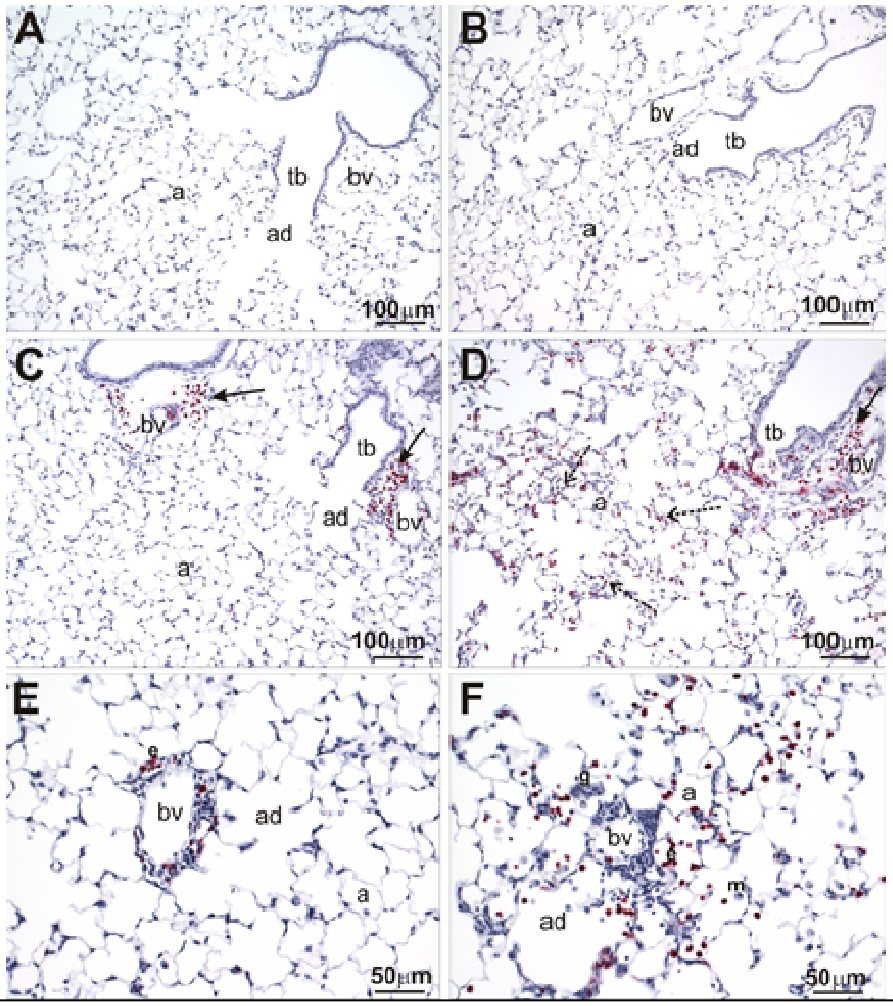


Figure 15 **Major Basic Protein (MBP) immunohistochemistry of HIF1α lungs**. Lung sections from HIF1α-sufficient (**A, C and E**) and PN4 HIF1α-deficient mice (**B, D and F**) following sensitization and challenge with saline (**A and B**) or OVA (**C, D, E and F**) were stained for MBP, an eosinophil-specific marker, via immunohistochemistry. Solid arrows indicate eosinophils located around terminal bronchioles and blood vessels while dashed arrows indicate eosinophils located out in the parenchyma (a = alveolus, bv = blood vessel, tb = terminal bronchiole, ad = alveolar duct).

and terminal bronchioles). The allergen-induced eosinophilic alveolitis was also reflected in the statistically significant increase in eosinophils and other inflammatory cells in the BALF from the PN4 HIF1α-deficient mice.

OVA sensitization and challenge in the HIF2α-sufficient and –deficient mice resulted in positive MBP staining, whereas saline treatment of these mice did not. However there was no difference in the amount of MBP staining between HIF2α-sufficient and HIF2α-deficient mice (Data not shown).

In the mice deficient in both HIF1α and HIF2α, the positively staining MBP cells in the OVA sensitized and challenged mice were found predominantly around the terminal bronchioles. There was no MBP staining in saline treated, nor in the parenchyma of the lung as seen previously in the PN4 HIF1α-deficient mice. It should be noted that, however, the loss of HIF1/2α severely decreased the amount of MBP staining in OVA treated mice (Figure 16).

3.8 Measuring total lung resistance in allergic airways

In addition to the histopathology data, treatment and HIF1α-related differences in total lung resistance were determined. In the control mice, methacholine-induced resistance was not statistically different between saline and OVA challenge, although a modest trend for OVA-induced enhancement was apparent (Figure 17). Following developmental recombination of HIF1α, the PN4 HIF1α-deficient, OVA-treated mice had the highest airway resistance of the treatment groups. At the highest dose of methacholine, the PN4 HIF1α-deficient, OVA-treated mice were significantly different

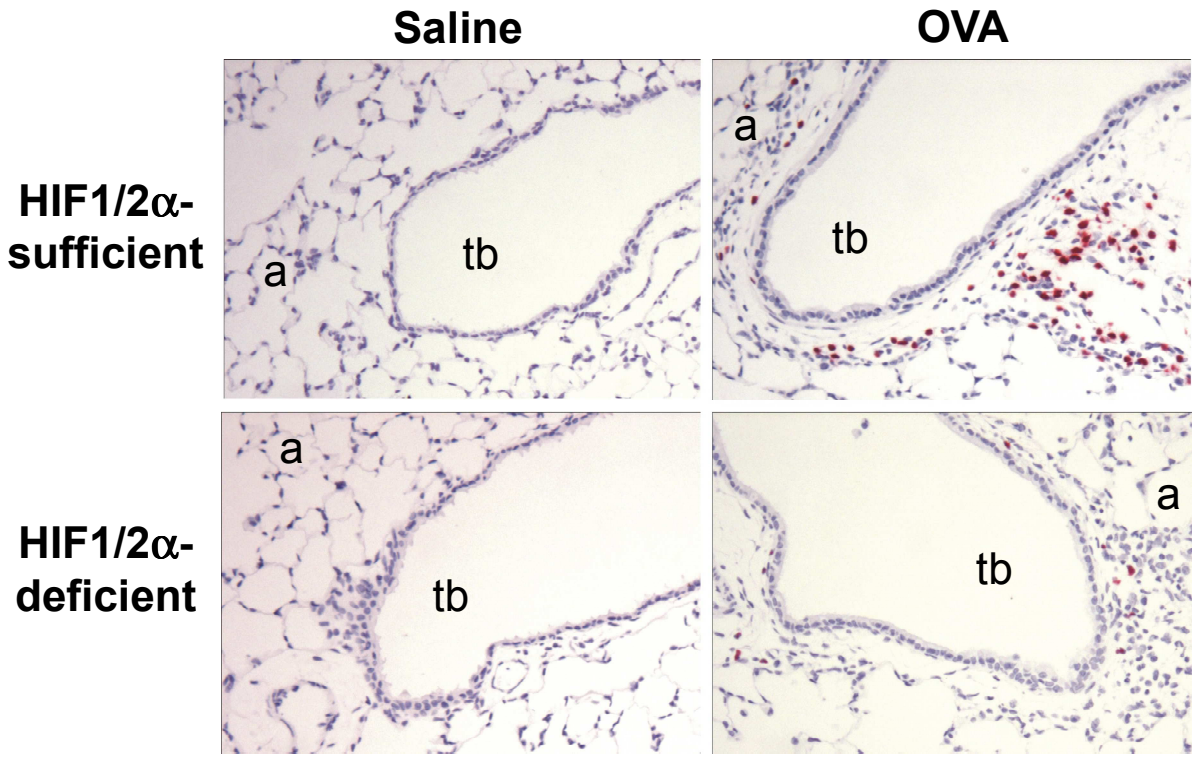


Figure 16 **Major basic protein (MBP) immunohistochemistry of HIF1/2α lungs**. Light photomicrographs of a centriacinar region from the lungs of HIF1/2α-sufficient (**top: left and right**) and HIF1/2α deficient (**bottom: left and right**) mice treated with saline (**left: top and bottom**) or OVA **(right: top and bottom**). Tissues were stained for MBP, an eosinophil-specific marker, via immunohistochemistry. (a = alveolus, tb = terminal bronchiole).

than control mice treated with OVA. At the 6, 12, 25 and 50 mg/ml methacholine doses, the PN4 HIF1α-deficient, OVA-treated group has a significantly higher airway resistance than their genotype controls.

Total lung resistance was also assessed in HIF2α-sufficient and HIF2α-deficient mice following saline and OVA treatment. Increasing doses of methacholine (3.125 to 50 mg/ml) were given methacholine-induced resistance was measured. Both the HIF2α-sufficient and HIF2α-deficient animals treated with saline displayed almost

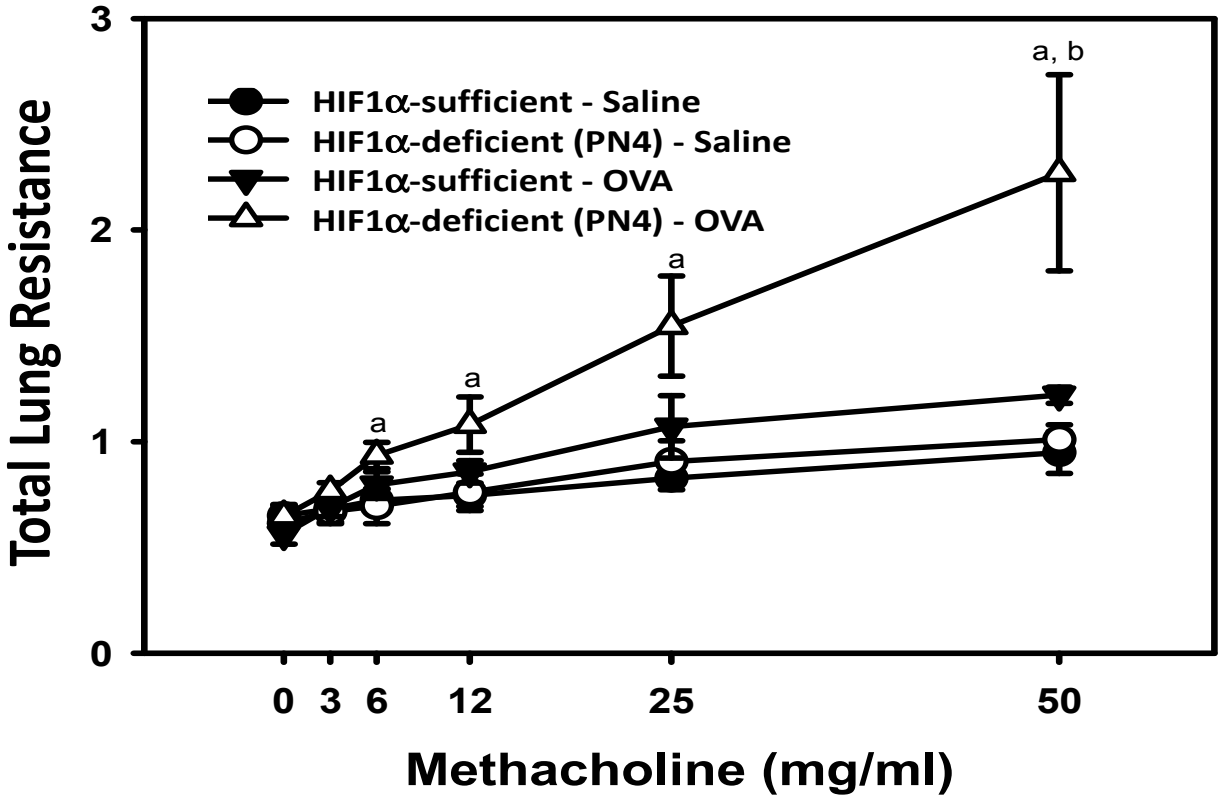


Figure 17 **Total lung resistance of HIF1α-sufficient and HIF1α-deficient mice**. Allergic airway resistance was measured using a Flexivent protocol in HIF1α-sufficient (**black**) and PN4 HIF1α-deficient (**white**) mice treated with saline (**circles**) or OVA (**triangles**). a = significantly different from HIF1α-deficient (PN4)/Saline group, b = significantly different from HIF1α-sufficient/OVA group.

identical total lung resistance. Both HIF2α-sufficient and HIF2α-deficient mice treated with OVA displayed increased total lung resistance when compared to their saline treated genotype controls. The only measurement that reached significance was the HIF2α-sufficient, OVA treated mouse at a 50 mg/ml methacholine dose. The HIF2α-deficient OVA treated mice showed a trend of having a slight decrease in total lung resistance when compared to the HIF2α-sufficient OVA treated group, though this difference did not reach significance (Figure 18).

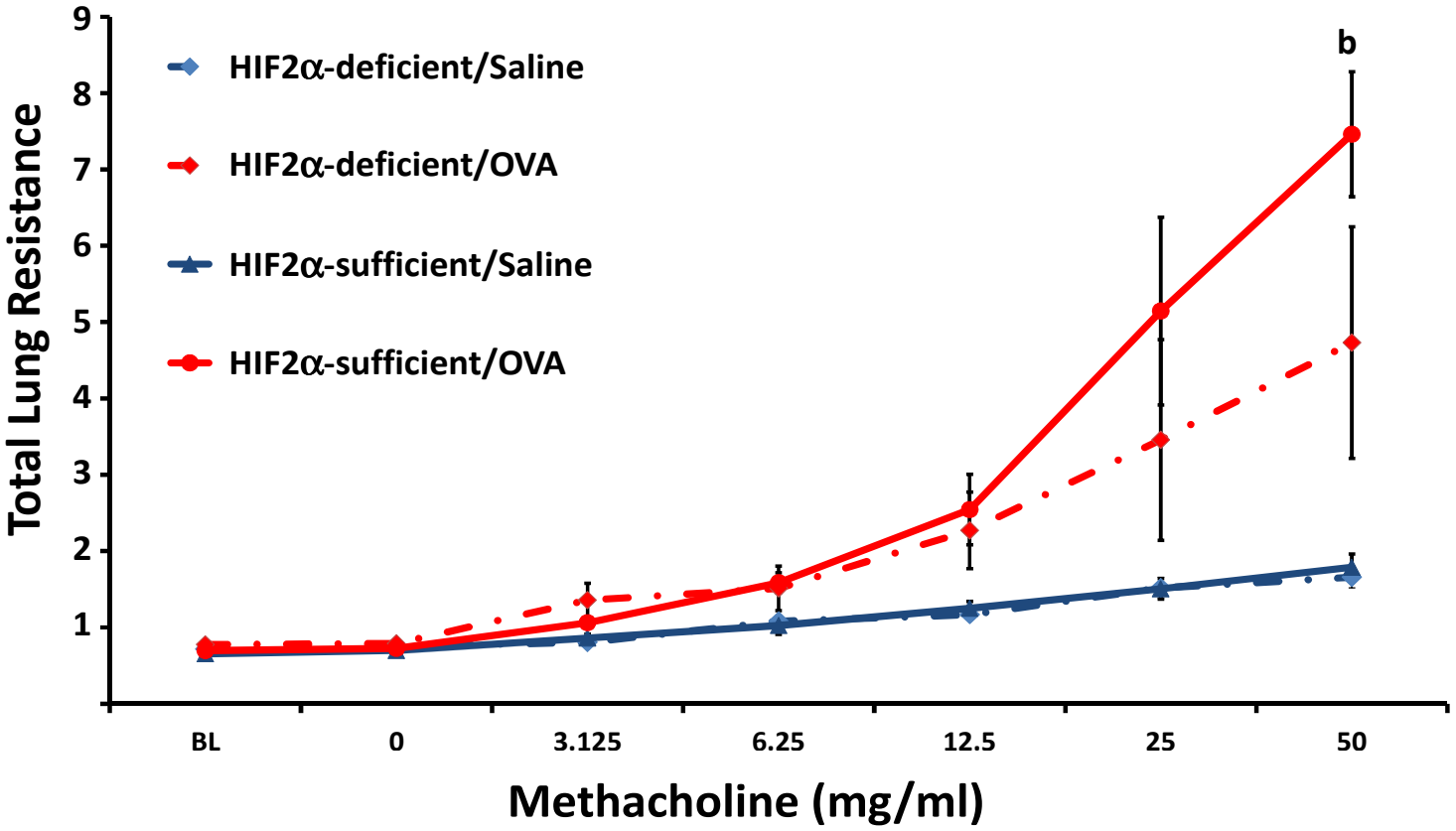


Figure 18 **Total lung resistance of HIF2α-sufficient and HIF2α-deficient mice**. Allergic airway resistance was measured using a Flexivent protocol in HIF2α-sufficient (**solid lines**) and HIF2α-deficient (**dashed lines**) mice treated with saline (**blue**) or OVA (**red**). Values are the mean +/- SEM. b = significantly different from saline treatment within genotype, ($P < 0.05$), ANOVA.

Finally, total lung resistance was also assessed in HIF1/2α-sufficient and HIF1/2α-deficient mice following saline and OVA treatment. Increasing doses of methacholine (3.125 to 50 mg/ml) were given and methacholine-induced resistance was measured. Typically, OVA treated mice display an increase in total lung resistance; however this was not the case for the HIF1/2α-sufficient and HIF1/2α-deficient mice. There were no statistically significant differences in total lung resistance among the groups (Figure 19).

3.9 Characterization of PN32 HIF1α-deficient mice.

It was necessary to determine if the timing of DOX was important for the observed change in immunity. To test this, mice were exposed to DOX after the post-natal development of the lung was completed. Administration of DOX for 10 days (PN32-PN42) was adequate to induce recombination and significantly reduce HIF1α levels in Clara and type II cells (Figure 20A). This level of recombination was similar to that observed in PN4 mice (Figure 6). In contrast with the PN4 HIF1α-deficient mice, the enhanced allergic inflammatory response in the lung, as compared to OVA-treated HIF1α-sufficient mice, was not evident in the PN32 HIF1α-deficient mice similarly treated with OVA. For example, a minimal mixed inflammatory cell infiltrate was present around the terminal bronchiole and associated pulmonary artery in the OVA-treated HIF1α-sufficient and -deficient PN32 mice. In contrast, there was

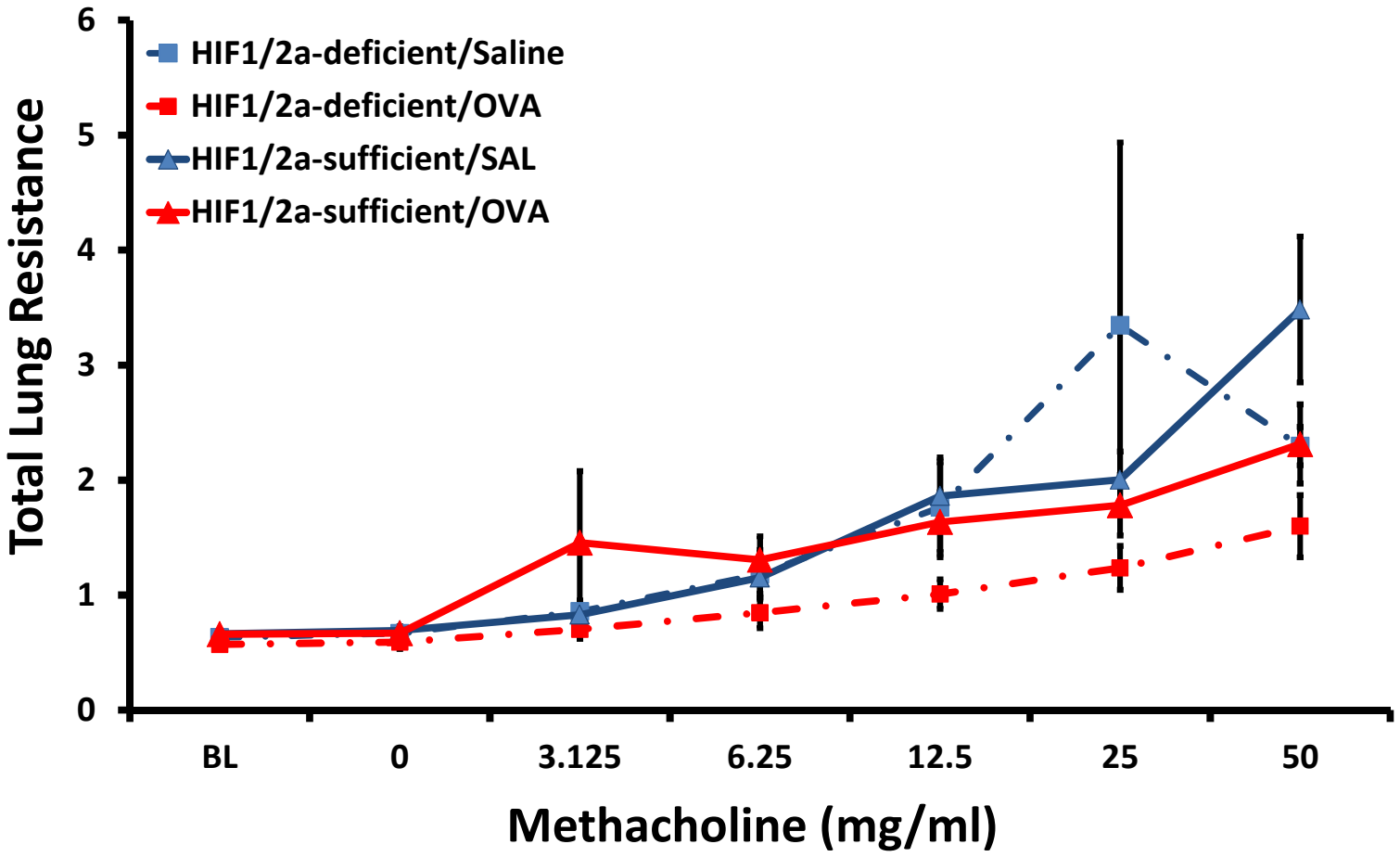


Figure 19 **Total lung resistance of HIF1/2α-sufficient and HIF1/2α-deficient mice**. Allergic airway resistance was measured using a Flexivent protocol in HIF1/2α-sufficient (**solid lines**) and HIF1/2α-deficient (**dashed lines**) mice treated with saline (**blue**) or OVA (**red**). Values are the mean +/- SEM.

marked peri-bronchiolar and –vascular inflammatory cell infiltrate in the OVA-treated HIF1α-deficient PN4 mouse. No centriacinar inflammation was present in the HIF1α-sufficient mouse.(Figure 17B).The total and differential cell counts in BALF and MBP staining confirmed the similar pattern of inflammation between HIF1α-sufficient and PN32 HIF1α-deficient OVA-treated mice (Figure 17C and D). The makeup of BALF in these PN32 HIF1α-deficient, OVA treated mice was approximately 50% eosinophils and 50% macrophages, which is not significantly different from OVA-treated, HIF1α-sufficient mice. Loss of HIF1α after completion of the alveolarization stage in the OVA model had no noticeable effect upon the tissues when analyzed via AB-PAS (data not shown).

The PN32 mice were exposed to DOX for 10 days. In contrast, the PN4 mice were exposed for 28 days and this difference might explain the lack of phenotype in the PN32 mice. To test this possibility, mice were exposed to DOX from PN4 to PN14. These PN4-14 mice displayed an exacerbated response to OVA treatment, similar to PN4 mice (Figure 20C). These results suggest that loss of HIF1α during the first two weeks of post-natal alveolarization impacts the innate immunity of the lung.

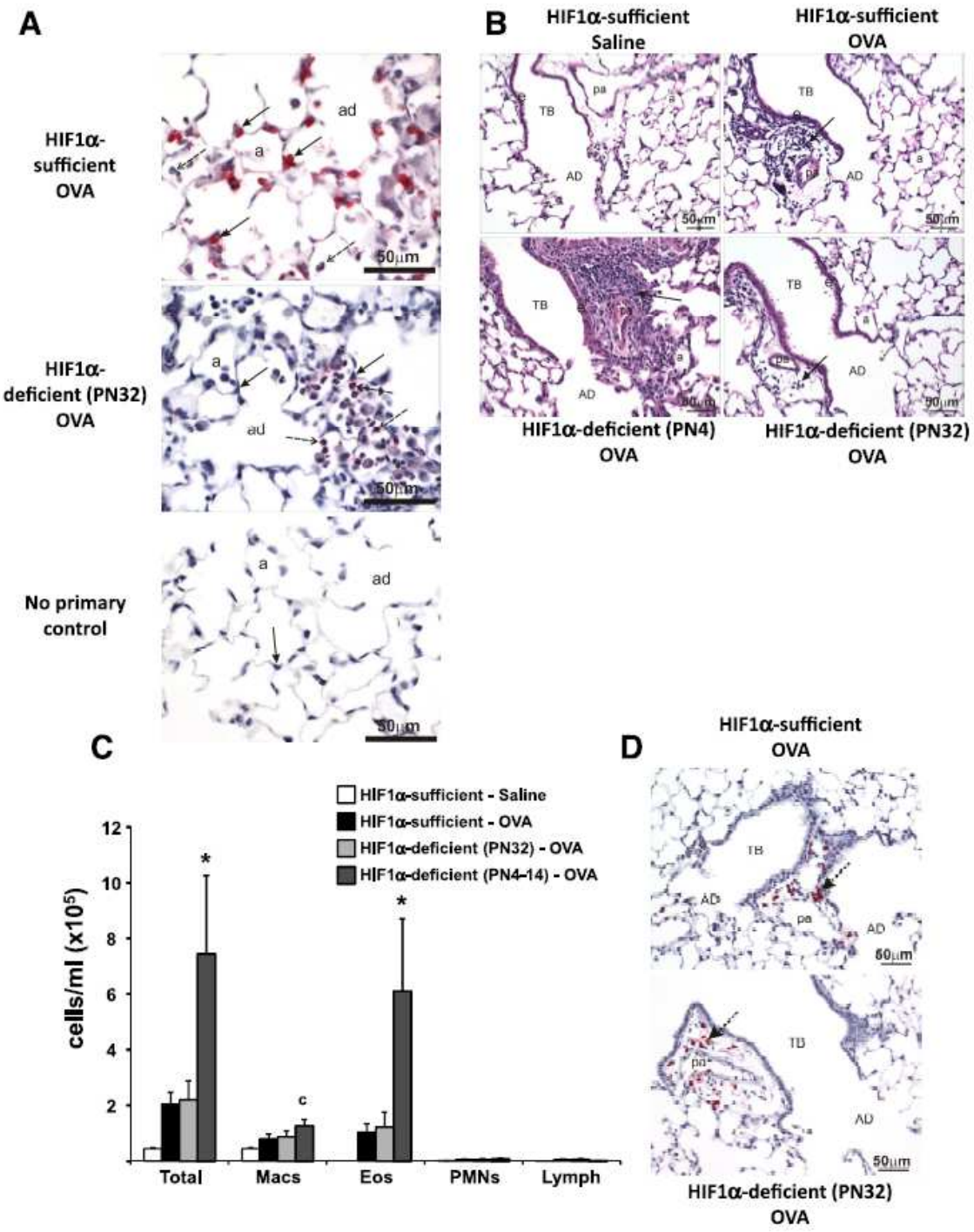


Figure 20 **HIF1α immunohistochemistry, H&E and BALF cellularity for PN32 and PN4-14 mice**. HIF1α immunohistochemistry of HIF1α-sufficient (**top**) and PN32 HIF1α–deficient (**middle**) mice after OVA sensitization and challenge. No primary control for HIF1α from a HIF1α-sufficient, saline treated mouse also pictured (**bottom**). Solid arrows indicate alveolar type II cells and dashed arrows indicate macrophages (**A**). Light photomicrographs of a centriacinar region

Figure 20 (cont'd) from the lungs of HIF1α-sufficient (**top left and right**) and HIF1α deficient PN4 (**bottom left**) and P32 (**bottom right**) mice treated with saline (**top left**) or OVA (**top right, bottom left and right**). Tissues were stained with hematoxylin and eosin. Arrows indicate areas of cellular infiltrate (**B**). HIF1α-sufficient (**white and black bars**), PN32 HIF1α-deficient (**hatched bars**), and PN4-14 HIF1α-deficient (**checked bars**) mice were sensitized/challenged with saline (**white bars**) or OVA (**black, hatched, and checked bars**). Total cells, as well as, the numbers of macrophages (Macs), eosinophils (Eos), neutrophils (PMNs), and lymphocytes (lymph) were determined from BALF (**C**). Values are the mean +/- SEM ($n \geq 7$). * = significantly different from all other groups $p < 0.05$, c = significantly different from saline treated. MBP immunohistochemistry performed on PN32 HIF1α-deficient (**bottom**) verifying the lack of a significant increase in eosinophils in these OVA-treated lungs as compared to HIF1α-sufficient (**top**) mice. Arrows indicate similar areas of eosinophilic infiltration surrounding the pulmonary artery (**D**). (ad = alveolar duct, a = alveolus, TB = terminal bronchiole, pa = associated pulmonary artery).

3.10 Determination of cytokines in BALF.

The cellular and histopathological results suggest that loss of HIF1α during postnatal development of the lung exacerbated OVA-induced inflammation, whereas loss after this developmental stage resembled control animals in response to OVA. To determine if these enhanced responses were correlated with increased production of Th2 type cytokines, the concentrations of IL-2, IL-4, IL-5, IFN-γ, TNF-α, IL-13, keratinocyte chemoattractant (KC), and IL-1β were analyzed from BALF. From this panel of cytokines, IL-4, IL-5, IL-13 and IL-1β showed significant differences between treatments or DOX exposure paradigms (Figure 21). IL-5 and IL-1β showed significant increases in BALF from OVA-treated PN4 HIF1α-deficient mice when compared to the OVA-treated HIF1α-sufficient controls. A similar pattern was not observed in the PN32 HIF1α-deficient mice. IL-4 showed a significant decrease in BALF from OVA-treated PN4 and PN32 HIF1α-deficient mice compared with the OVA-treated HIF1α-sufficient mice. In contrast, IL-13 showed a significant increases in BALF of both HIF1α-sufficient and PN4 HIF1α-deficient OVA treated mice compared with their respective saline controls. This pattern was replicated in the PN32 HIF1α-deficient mice; however, the levels did not reach significance. Taken together, these results suggest that the enhanced inflammation observed following deletion of HIF1α is dependent upon the developmental timing of the deletion, and this enhanced response is evident at the level of cellular infiltrate, functional responses, and cytokine production.

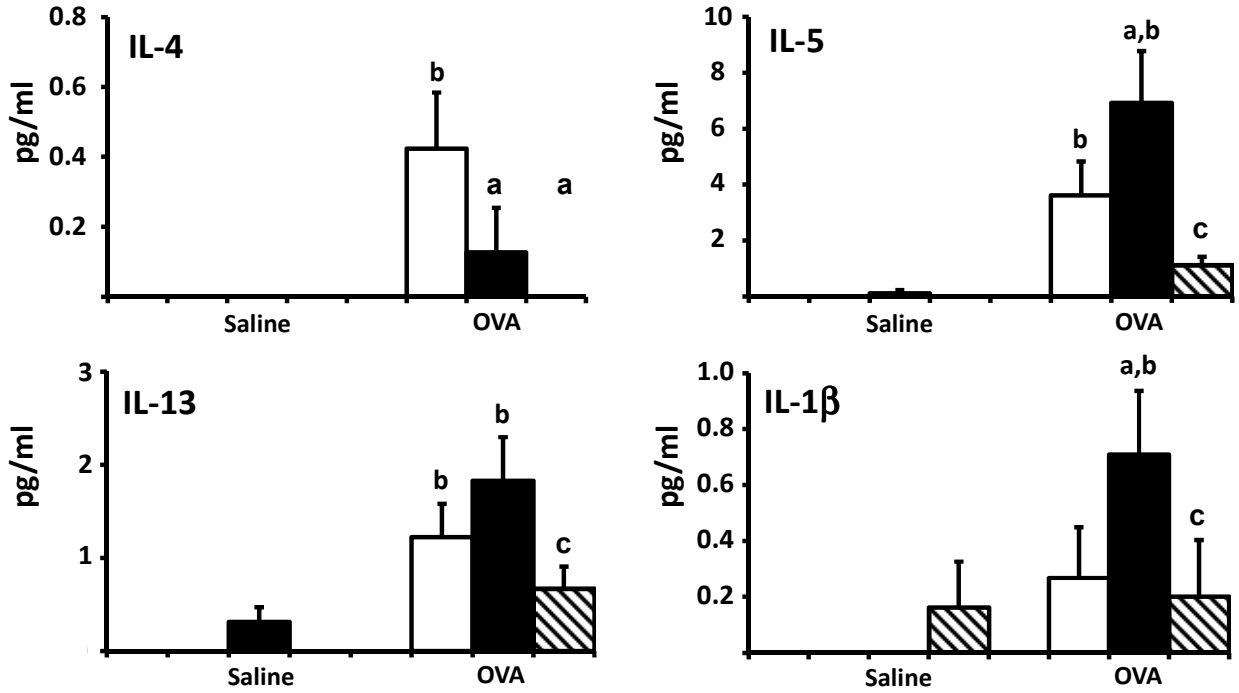


Figure 21 **The effect of OVA on cytokine levels**. Cytokine levels in BALF were determined using a Th1/Th2 bead array panel (BD Bioscience). Samples were collected from HIF1α-sufficient (**white bars**), PN4 HIF1α-deficient (**black bars**) and PN32 HIF1α-deficient (**hatched bars**) mice sensitized/challenged with saline or with OVA. Data are represented as the mean +/- SEM ($n \geq 9$). a = significantly different from HIF1α-sufficient within treatment, $p < 0.05$, b = significantly different from saline treatment within genotype, $p < 0.05$, c = significantly different from PN4 HIF1α-deficient within treatment, $p < 0.05$.

3.11 BALF cellularity after OVA sensitization and OVA challenge concurrent with DMOG dosing

After determining that the loss of HIF1α in the lung-specific, inducible mouse model caused an exacerbated response to OVA, it was hypothesized that perhaps the activation of HIF may offer protection. Dimethyloxalylglycine (DMOG) is a cell permeable competitive inhibitor of the prolyl hydroxylase which modifies HIFα to signal

its degradation. Therefore, dosing with DMOG will lead to HIFα stabilization, among other things. Upon sensitization of mice with OVA and challenge concurrent with DMOG dosing, BALF was analyzed. Minimal amounts of total cells were found in the BALF of saline treated animals, both control and DMOG treated. Animals treated with OVA alone only had minimal increases in total cells, however animals treated concurrently with OVA and DMOG had significantly higher total cells than all other groups (Figure 22A). When the differential cells counts were performed, it was determined that this increase in total cells was due to an increase in eosinophils, also only in the animal group treated with both OVA and DMOG (Figure 22B). All other differential cell types (macrophages, lymphocytes and neutrophils) were not statistically different (Data not shown).

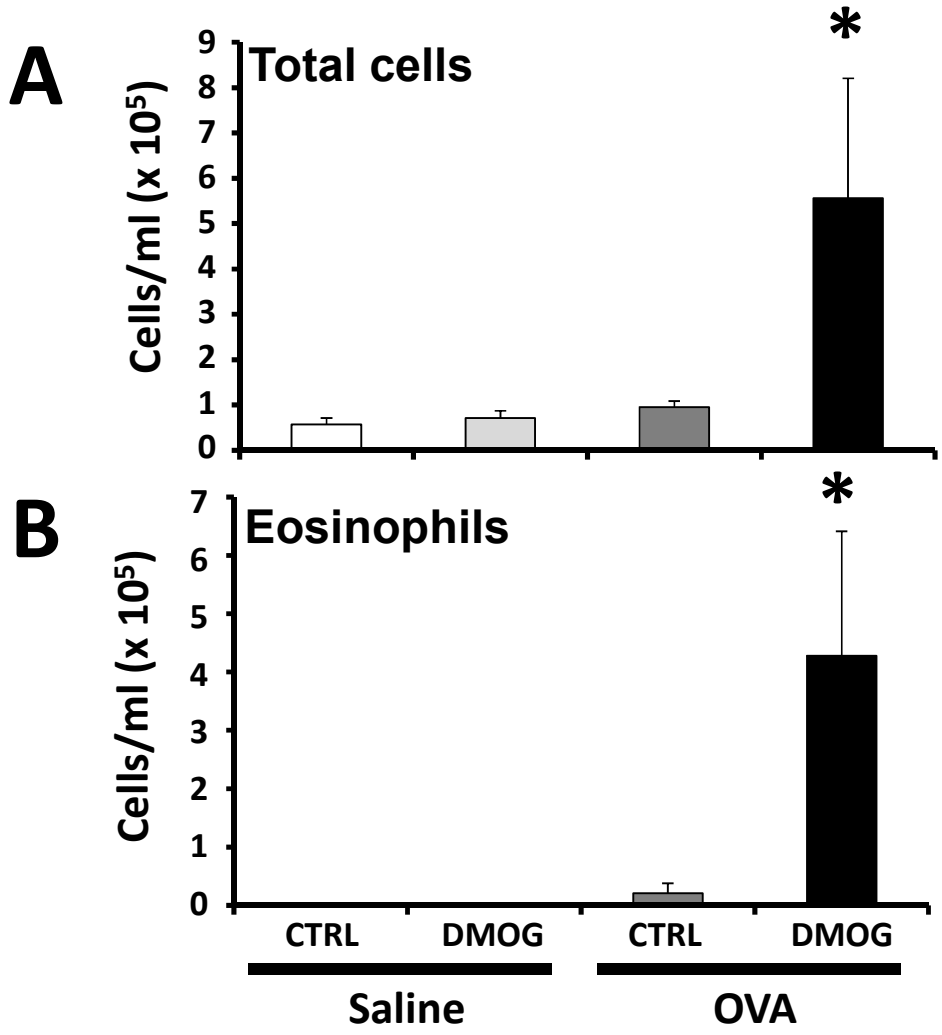


Figure 22 **The effect of DMOG on OVA-induced BALF cellularity**. Total cells (**A**) and eosinophils (**B**) were analyzed in the BALF of animals sensitized and challenged with saline or OVA, in the absence (Ctrl) or presence of the PHD inhibitor, dimethyloxalylglycine (DMOG). Values are the mean +/- SEM. * = significantly different from all other groups. $p<0.05$, ANOVA.

3.12 BALF cellularity after OVA sensitization and challenge in UBC mice

After finding that the loss of HIF1α in the lung-specific, inducible mouse model causes an exacerbated response to OVA, it was hypothesized that if HIF1α was lost in

the whole animal and not just in the lung epithelial cells, there may be an even further exacerbation of the inflammatory response. To test this hypothesis, the UBC-Cre-ERT2$^{+/-}$/HIF-1$\alpha^{fl/fl}$ mice were used. In this mouse model, tamoxifen (TAM) is capable of inducing functional deletion of HIF1α in all cells of the body. After the OVA sensitization and challenge protocol, in the presence and absence of TAM, BALF was analyzed. Minimal and equal amounts of total cells were found in the saline treated groups, both UBC HIF1α-sufficient and UBC HIF1α-deficient. Upon OVA treatment, both UBC HIF1α-sufficient and –deficient animals had increases in total cells, with UBC HIF1α-sufficient animals seeming to have a higher increase, though neither reached significance (Figure 23A). When analyzing the differential cell counts, there were significantly fewer eosinophils in the UBC HIF1α-deficient, OVA treated group compared to the UBC HIF1α-sufficient, OVA treated group (Figure 23B). All other differential cell types (macrophages, lymphocytes and neutrophils) were not statistically different (Data not shown).

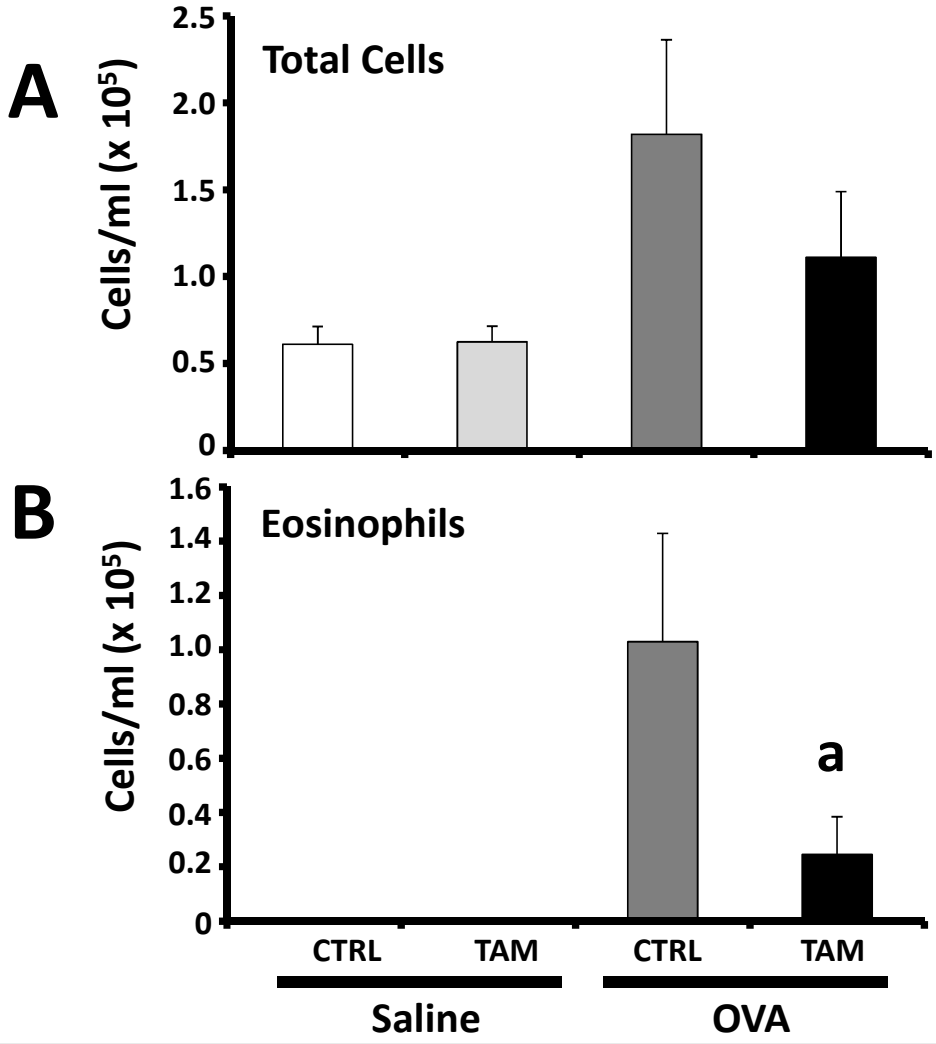


Figure 23 **The effect of OVA on BALF cellularity in UBC mice**. Total cells (**A**) and eosinophils (**B**) were analyzed in the BALF of animals sensitized and challenged with saline or OVA, in the absence (Ctrl) or presence of the the Cre-ERT2 inducer, tamoxifen (TAM). Values are the mean +/- SEM. * = significantly different from all other groups. $p<0.05$, ANOVA.

3.13 BALF cellularity of hyperoxia mice after OVA sensitization and challenge

The observation that loss of HIF1α impacts lung immunity has strong clinical relevance, especially when premature birth is considered. First, the data presented so far, demonstrate that loss of HIF1a during early, post-natal development, biases the

lung towards a Th2-mediated inflammation. Second, premature birth exposes the lungs of the infant to higher than normal (i.e. hyperoxia) prior to the tissue being capable of coping with the stress, and in many cases these infants require supplemental oxygen, (i.e more hyperoxic stress) for survival. Third, premature infants are predisposed to atopic diseases, such as asthma, and this increased susceptibility is further exacerbated upon supplemental oxygen therapy [84, 94]. Finally, hyperoxia can inhibit normal HIF1α-mediated signaling by maintaining PHD activity. Taken together, these results suggest that HIF1α plays a role in the link between premature birth and asthma susceptibility. To test this link, mice were exposed from PN0 to PN14 to hyperoxia (75% O_2). Control animals were exposed to room air. Following this modulation in O_2 exposure, animals were allowed to reach ~PN38 when the OVA sensitization and challenge protocol began. BALF from these animals was analyzed, and the two weeks of exposure to hyperoxia led to slight increases in total cells. More importantly, hyperoxia led to a significant increase in eosinophils compared to room air exposed mice (Figure 24).

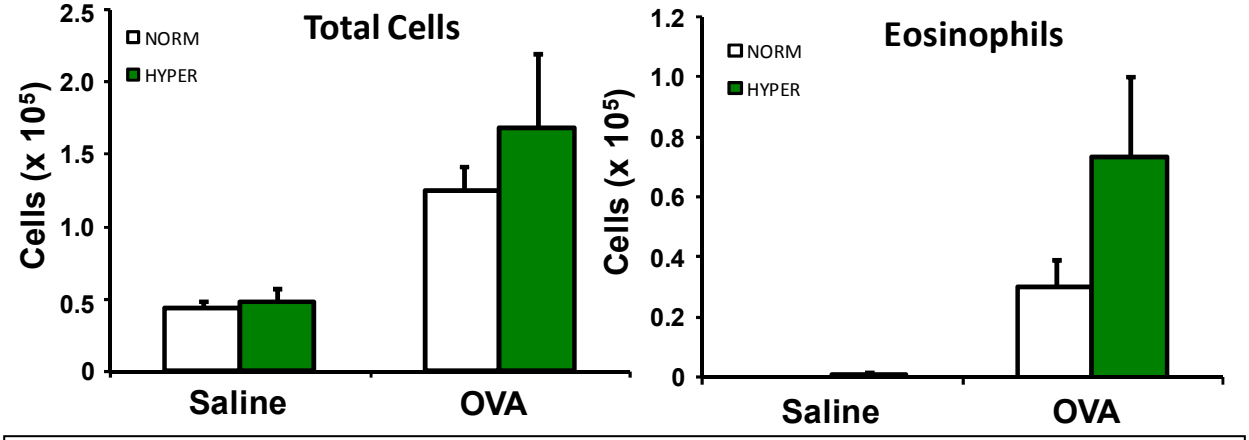


Figure 24 **Comparison of total cell and eosinophil counts from control and hyperoxia exposed mice**. Mice were exposed to room air (**white bars, NORM**) or 75% O_2 (**green bars, HYPER**) from PN0 to PN14. Mice were then allowed to grow until they were 6 weeks of age under normal conditions. At that time, they were exposed to saline or OVA as described in the methods. Following challenge, mice were sacrificed and cellularity of the BALF was determined.

3.14 Hyperoxia-induced changes in lung architecture and HIF1α immunohistochemistry

To determine if the 14 day, 75% O_2 exposure impacted the post-natal development of the lung, H&E staining was performed. Mice exposed to room air displayed normal architecture. In contrast, the hyperoxia-exposed mice displayed a phenotype similar to bronchopulmonary dysplasia (BPD, Figure 25). These mice displayed increased alveolar size and thin septa. It should also be noted that these hyperoxia-exposed mice appeared smaller and weighed less than their room air exposed counterparts, but this did not reach significance (Data not shown).

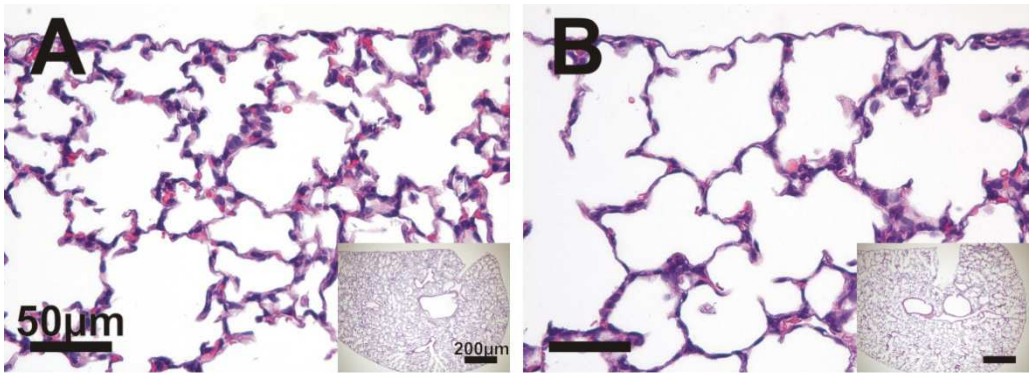


Figure 25 **H&E staining of control and hyperoxia exposed lungs**. Mice were exposed to room air (**A**) or hyperoxia (75% O_2, **B**) for 14 days and lung tissue was extracted. Tissue slices were prepared and stained with H&E. Figures are 400X magnification with 40X magnification on inset.

The model suggests that hyperoxia leads to a Th2 bias through inhibiting HIF1α during this critical postnatal development. To determine if the hyperoxia exposure led to a decrease in HIF1α protein within the lung, immunohistochemistry was performed on room air and hyperoxia exposed lungs, fixed at PN14, while still exposed to 75% O_2. There was a decrease in HIF1α-positive staining in the bronchial artery smooth muscle cells and epithelial cells lining the bronchial airway. There was also a slight but reproducible decrease in the parenchymal epithelium (Figure 26). The modest decrease in staining is most likely due to the time it takes to perform the necropsy efficiently within the confines of the glove box. These results, however, support the hypothesis that hyperoxia phenotypically copies early postnatal deletion of HIF1α and hyperoxia decreases HIF1α levels in the lung.

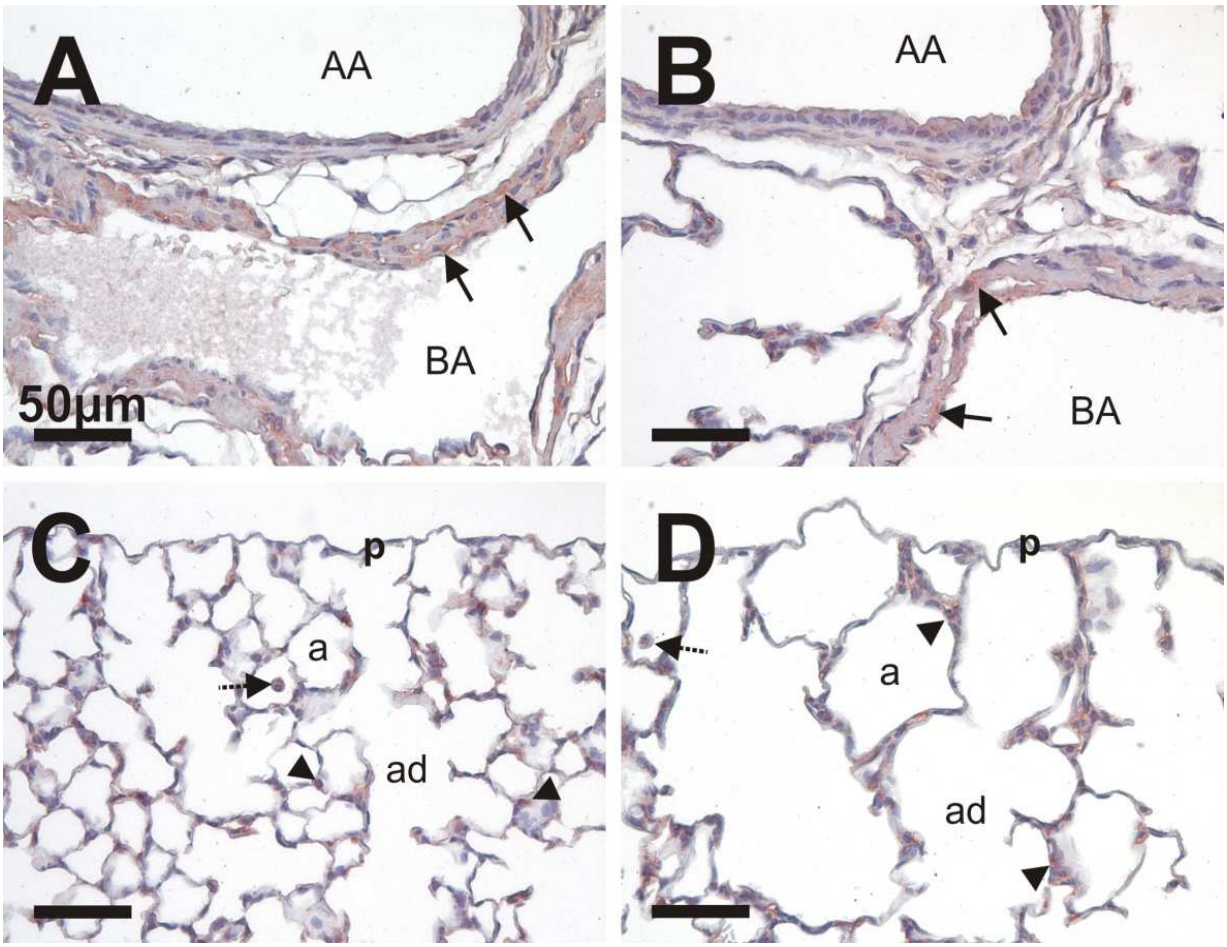


Figure 26 **HIF1α immunohistochemistry**. Mice were exposed to room air (**A and C**) or hyperoxia (75% O_2, **B and D**) for 14 days and lung tissue extracted. Tissue slices were prepared and analyzed by immunohistochemistry using a HIF1α-specific antibody. A decrease in overall HIF1α positive staining was observed in the bronchial artery smooth muscle cells (**solid arrows**) (**A and B**) ATII cells (**arrowheads**) and alveolar macrophages (**dashed arrows**) (**C and D**). AA = axial airway, BA = bronchial artery, ad = alveolar duct, a = alveolus.

3.15 Hyperoxia-induced changes in Galectin-3 and IL-1R2 immunohistochemistry

To determine if the 14 day, 75% O2 exposure was modulating similar signaling pathways as the lung-specific, inducible mouse model, immunohistochemistry was performed for the expression of epithelial-derived factors that are linked to changes in tissue immunity, Galectin-3, and IL-1R2. Compared to control mice, Galectin-3 staining

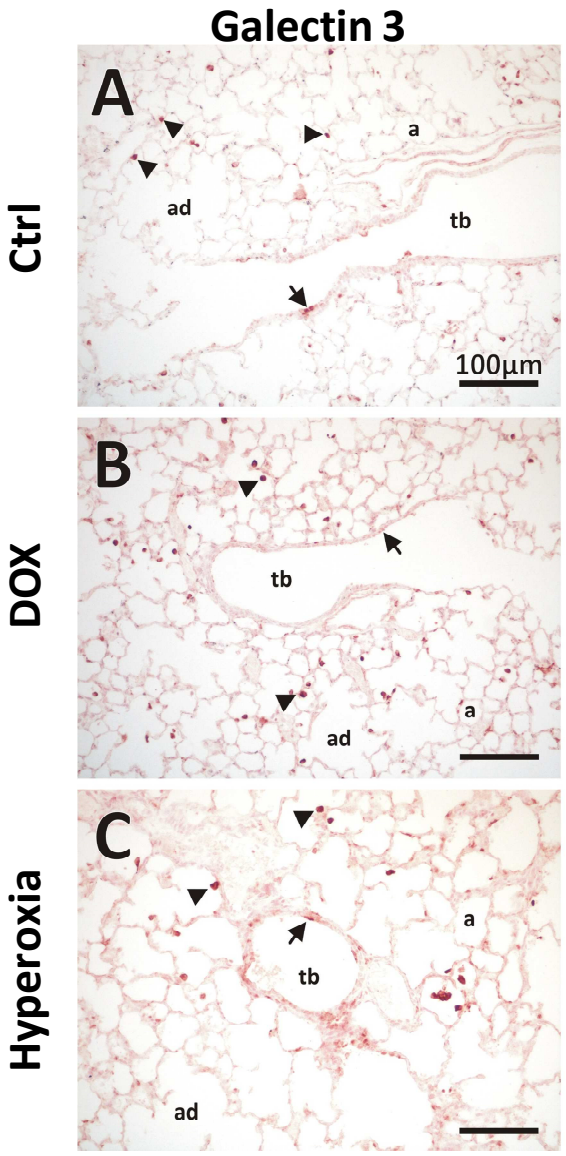


Figure 27 **Galectin-3 immunohistochemistry**. Mice were exposed to room air (**A**), DOX (**B**), or hyperoxia (75% O_2, **C**) from PN0-14 and lung tissue was extracted. Tissue slices were prepared and analyzed by immunohistochemistry using a Galectin-3-specific antibody. Positive Galectin-3 staining is seen in the bronchial artery smooth muscle cells (**solid arrows**) and ATII cells (**arrowheads**). tb = terminal bronchiole, ad = alveolar duct, a = alveolus.

increased in the HIF1α-deficient mice compared to controls (Figure 27A and B). This DOX-induced increase in galectin-3 staining was also observed in the mice exposed to hyperoxia (75% O_2) during the first two weeks of life. (Figure 27B and C).

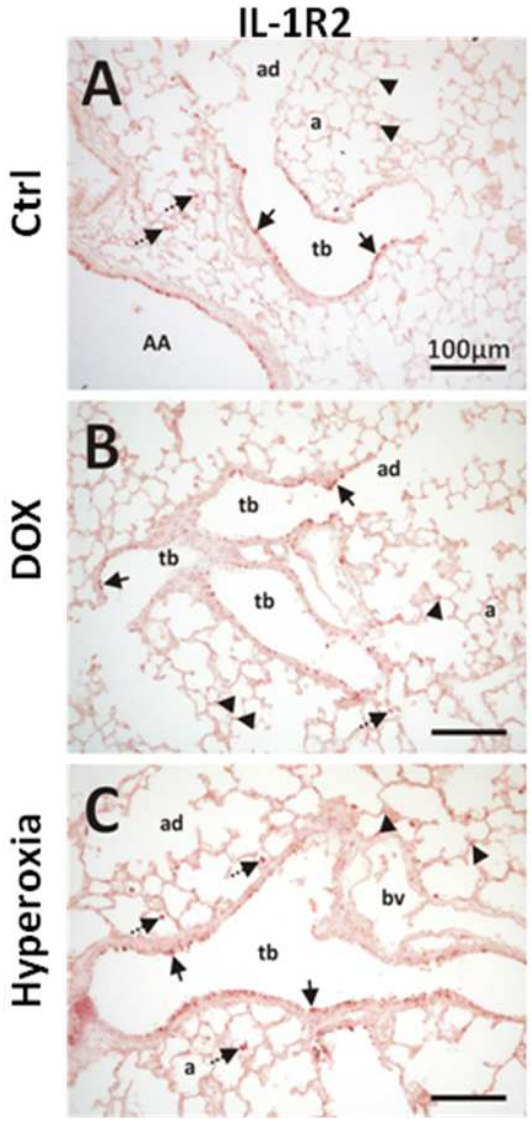


Figure 28 **IL-1R2 immunohistochemistry**. Mice were exposed to room air (**A**), DOX (**B**), or hyperoxia (75% O_2, **C**) from PN4-14 and lung tissue was extracted. Tissue slices were prepared and analyzed by immunohistochemistry using an Il-1R2-specific antibody. Positive IL-1R2 staining is seen in the bronchial artery smooth muscle cells (**solid arrows**), ATII cells (**arrowheads**), or alveolar macrophages (**dashed arrows**). tb = terminal bronchiole, ad = alveolar duct, a = alveolus.

IL-1R2 is a soluble IL-1 receptor, capable of acting as a decoy for the membrane bound version, and is thus capable of inhibiting normal IL-1 signaling. Analysis of IL-1R2 staining in DOX-treated mice showed a similar pattern of expression to that of galectin-3 (i.e. increased in the HIF1a-deficient mice compared to controls) (Figure 28A and B). In addition, this increased IL-1R2 staining was also observed in the hyperoxia treatment (Figure 28C).

3.16 Dendritic cell subpopulations at 1 week and 6 weeks

The developmental timing of DOX and its relation to the phenotypic change in inflammatory response to allergen suggests that HIF1α plays a role in programming the immunity of the lung. One possible explanation for this phenotype is a change in key inflammatory cell types within the lung. The most prominent antigen presenting cells in the lung are the dendritic cells. To determine if early developmental exposure to DOX (PN4) was capable of altering the population of dendritic cells in the lung, flow cytometry

	pDC	mDC	Other DC
CD11c	+	+	+
CD11b	+	-	-
B220	-	+	-

Figure 29 **Flow cytometry experimental design**. All cells were first gated on CD11c+ status. From there, they were categorized into pDC, mDC or other DC groups based on their CD11b and B220 status. Activation status was determined with the MHC II marker.

experiments were performed 1 week and 6 week time points. The 1 week group consisted of mice that had 1 week of DOX treatment, while the 6 week group had received the full DOX treatment (PN4-28). The surface staining markers that were used in this experiment were as follows: CD11c, CD11b, B220, F4/80 and MHC II. Only cells expressing CD11c were gated on, since this transmembrane protein is found at high levels on dendritic cells. After gating for CD11c, CD11b and B220 were used to determine classification as pDC, mDC or 'other' DC (Figure 29). The MHC II marker was used to indicate an 'activated' or 'non-activated' status of the DCs. Analysis of 1 week and 6 week DOX groups are categorized as either mDC, pDC, or 'other' DC, as well as categorized as either 'non-activated' or 'activated' (Figure 30).

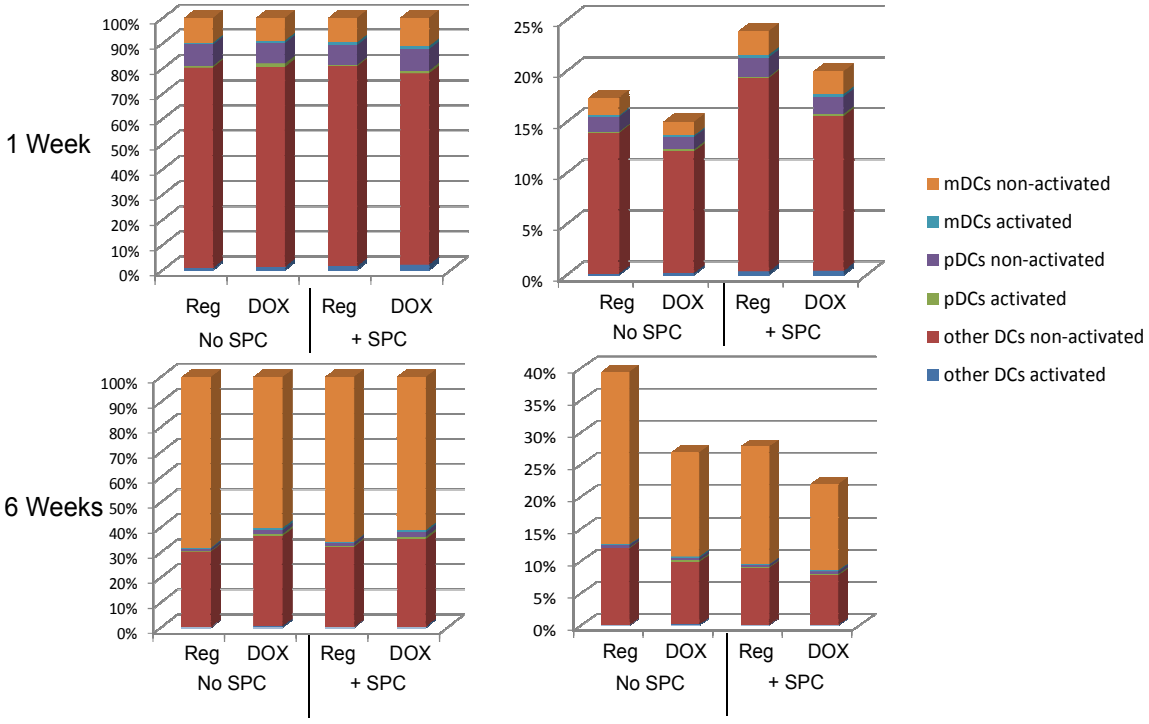


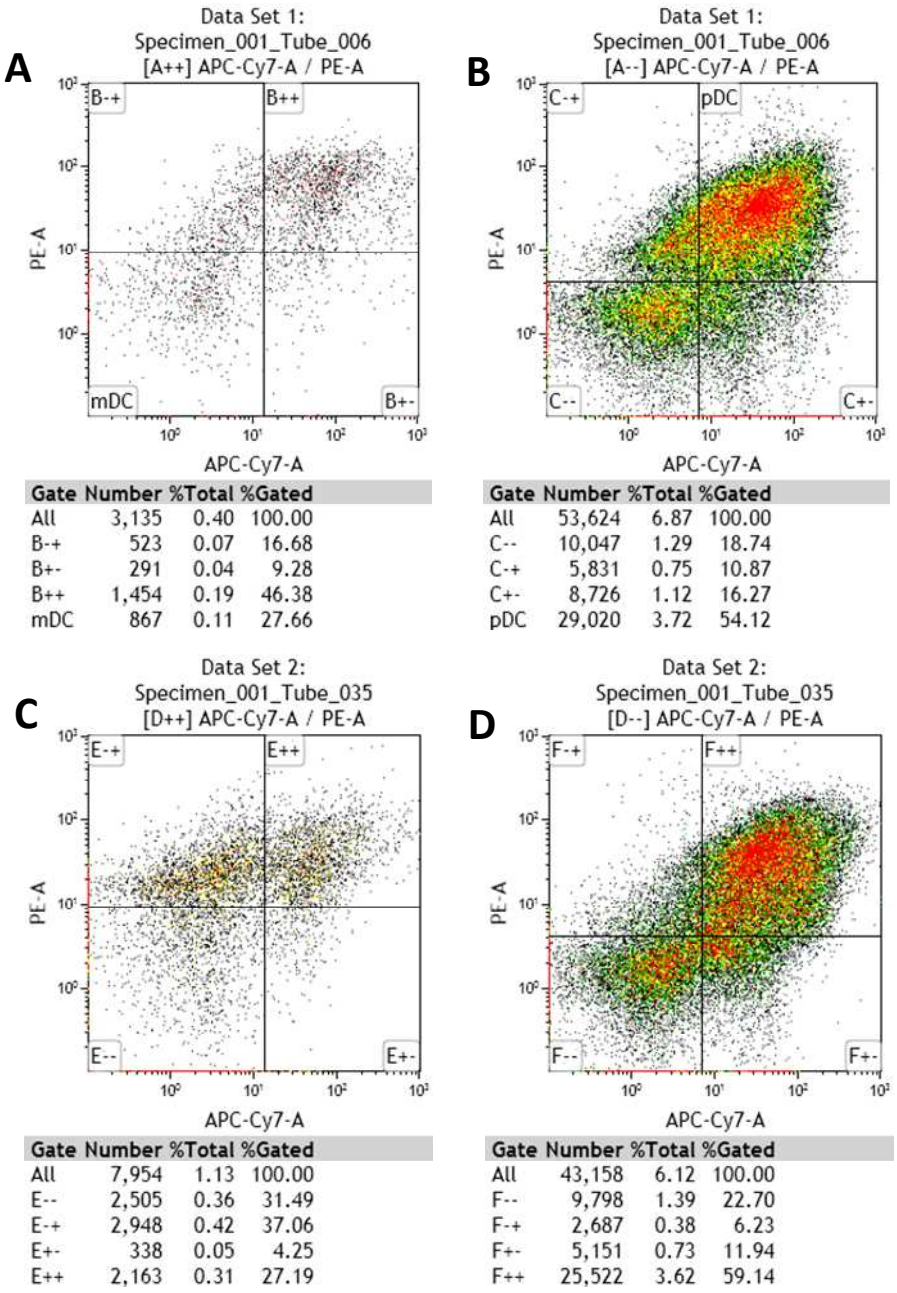
Figure 30 **Classes of DCs found in 1 week and 6 week mouse lungs**. Graphs on the left represent percentages based on the total percentage of DCs analyzed. Graphs on the right represent percentages based on the number of total cells analyzed. There were no significant differences in DC populations when comparing HIF1α-deficient (DOX, +SPC) with HIF1α-sufficient (Reg, -SPC; Reg, +SPC; DOX, -SPC) groups.

3.17 Dendritic cell populations after 10 days of DOX

Another flow cytometry experiment was performed to help determine the role of different types of DCs in our mouse model. This experiment was performed after 10 days of DOX treatment. Half of the litters in the experiment were treated with DOX beginning on PN4 until sacrifice on PN15, while the other half of the litters were maintained on regular food and water until sacrifice on PN15. The surface markers used for this experiment were based on a paper by Whitsett et al [95] and included the following: CD11c, CD11b, Gr-1, CD317 and CD86. Cells that were CD11c positive, CD11b positive, Gr-1 negative and CD317 negative were classified as mDCs while cells that were CD11c low, CD11b negative, Gr-1 positive and CD317 positive were considered pDCs (Figure 31). These results do confirm the previous results and suggest a decrease in the mDC population upon loss of HIF1α (Figure 32).

	pDC	mDC
CD11c	low	+
CD11b	-	+
Gr-1	+	-
CD317	+	-

Figure 31 **Flow cytometry experimental design**. Cells were gated on CD11c low, CD11b negative, Gr-1 positive, and CD317 positive and called pDCs. Cells were gated on CD11c positive, CD11b positive, Gr-1 negative and CD317 negative and called mDCs.



Gate	Number	%Total	%Gated
All	3,135	0.40	100.00
B-+	523	0.07	16.68
B+-	291	0.04	9.28
B++	1,454	0.19	46.38
mDC	867	0.11	27.66

Gate	Number	%Total	%Gated
All	53,624	6.87	100.00
C--	10,047	1.29	18.74
C-+	5,831	0.75	10.87
C+-	8,726	1.12	16.27
pDC	29,020	3.72	54.12

Gate	Number	%Total	%Gated
All	7,954	1.13	100.00
E--	2,505	0.36	31.49
E-+	2,948	0.42	37.06
E+-	338	0.05	4.25
E++	2,163	0.31	27.19

Gate	Number	%Total	%Gated
All	43,158	6.12	100.00
F--	9,798	1.39	22.70
F-+	2,687	0.38	6.23
F+-	5,151	0.73	11.94
F++	25,522	3.62	59.14

Figure 32 **Classes of DCs found in PN15 mice with and without 10 days DOX treatment**. Cells were gated to determine mDC (**A and C**) and pDC (**B and D**) subpopulations from HIF1α-sufficient mice (**A and B**) and HIF1α-deficient mice (**C and D**). Percentage of mDCs are essentially identical between groups (27.66% vs 27.19%), while pDCs showed a slight difference (54.12% vs 59.14%).

CHAPTER FOUR

Discussion

Asthma is a complex disease that involves interactions between the various cell types of the lung, the cytokines they produce, as well as the genetics and other predisposition factors of the individual. Allergic asthma is characterized by various pulmonary pathologic features including eosinophilic inflammation in tracheobronchial airways, mucus cell metaplasia of airway epithelium, airway hyperreactivity, and the presence of certain cytokines related to a Th2-mediated response. Though recent literature has demonstrated a role for hypoxia and HIF-mediated signaling in inflammation, little is known about their role in the etiology of asthma [96-98]. The results presented here suggest that HIF1α, specifically in the epithelial cells of the lung, plays a central role in establishing the immunity of the lung. Loss of HIF1α led to an enhanced response to OVA sensitization and challenge. This was evidenced by the significant increase in eosinophils and airway hyperreactivity, as well as the extension of the eosinopilic inflammatory response into the proximal alveolar ducts and adjacent alveolar parenchyma deep in the lung. This exacerbation of the inflammatory and functional responses did not include a significant increase in mucus cell metaplasia, suggesting the signal responsible for this phenotype is HIF1α-independent. It was also shown that this exacerbation is specific to HIF1α, as the HIF2α-deficient mice did not show the same phenotype. Additionally, HIF1/2α-deficient mice seemed to actually show a reduced inflammatory response as evidenced by a decrease in total cells in the BALF, specifically a reduction in eosinophils, a decrease in MBP staining and a visual decrease in cellular infiltrates seen in H&E stained lung sections. Finally, the phenotypic changes of exacerbation in the HIF1α-deficient mice were dependent upon

the developmental timing of HIF1α deletion, but not dependent on length of DOX treatment.

The interesting lack of an eosinophil response as seen in the PN32 mice in comparison to the PN4 mice suggests that HIF1α derived from the alveolar epithelium, Clara and ATII cells specifically, is required during early postnatal development of the lung to establish the proper immune responses of tissues to allergens. Our findings suggest that early events in postnatal development induce HIF1α to direct proper immune maturation. For example, early exposure to inhaled bacteria and allergens will induce inflammation that can lead to HIF1α signaling via changes in local oxygen levels and the production of cytokines [99-101]. As such, HIF1α signaling might contribute to airway immune development during early-life exposure to pathogens. In a model of influenza, it has been shown that neonatal hyperoxia exacerbates the response of the mouse to influenza as an adult [102]. This was apparent by increased leukocyte recruitment to the lungs and increased pro-inflammatory cytokines, similar to what was observed in the P4 HIF1α-deficient mice.

Recently, the role of the epithelium in allergen-induced airway disease has started gaining attention [103, 104]. This interest is driven, at least in part, by the variability or lack of efficacy of treatments that target the inflammatory cells [105-108]. These conflicting findings suggest that targeting inflammatory cells, such as eosinphils, is not productive for a broad range of asthmatics, and other cell types must be considered. If it is viewed in this way, it might be possible to target HIF1α signaling

early in development for at-risk patients, such as preterm infants, and lessen the severity of allergic airway diseases.

In other studies, mice deficient in hypoxia signaling are partially protected from OVA-induced inflammation [109, 110]. In these models, the deficiency in hypoxia-mediated signaling occurs in all of the cells of the mouse, including macrophages and other myeloid cells (any blood cell that is not a lymphocyte). Myeloid cells can either be resident at the locations of inflammation, or they can be recruited to needed areas. During inflammation, up to 95% of inflammatory cells that are present have been recruited and are not resident to those areas [111]. Therefore, eosinophils, macrophages, mDCs and other myeloid cells need to move into areas of inflammation where localized hypoxia usually occurs. It has been shown that macrophages require HIF1α for proper function and this function plays a role in allergen-induced airway disease [96, 112]. These studies confirm the results of both the UBC HIF1α-deficient mice and the DMOG treated mice. In the UBC HIF1α-deficient model, HIF1α is being removed from all cells in the body, including all inflammatory cells, so it is no surprise that the UBC HIF1α-deficient mice showed fewer total cells and fewer eosinophils after OVA sensitization and challenge. Additionally, in the DMOG treated mice, HIFs everywhere, including in the inflammatory cells, are being stabilized. The increased inflammatory response in this model is also consistent with the idea that HIF1α activation is necessary for normal immune cell function, and inappropriate HIF activation can enhance the inflammatory response. In contrast, the lung-epithelial specific HIF1α-deficient mice described here have targeted deficiencies in Clara and ATII cells only. These cells act as a signaling mediator between macrophages and dendritic cells and

are critical to the progression of airway disease and remodeling [113]. Taken together, these results suggest that global loss of HIF1α protects against allergen-induced lung damage, most likely through inhibiting HIF1α signaling in inflammatory cells. Global stabilization of HIFs results in an increased inflammatory response, most likely due to stabilizing HIFs in inflammatory cells. In contrast, specific loss from alveolar epithelium during postnatal development results in an increased allergic airway hyperreactivity phenotype.

The opposite signaling cascade would be true for oxygen-dependent preterm infants. There is extensive literature linking premature birth and an increased asthma susceptibility [114-116]. This link between prematurity and asthma is complicated and involves many different facets, including abnormal lung development and cellular differentiation, bronchopulmonary dysplasia, and abrupt and severe changes in the oxygen exposure to the lungs of the neonate. Although complicated, it is interesting to hypothesize that the premature birth will expose the lungs to ambient levels of oxygen prior to the maturation of the tissue. This premature hyperoxia might inhibit normal HIF1α-mediated signaling that is essential for normal fetal lung development [87, 117]. Moreover, this hyperoxic environment, especially in those infants placed on ventilators with oxygen levels higher than ambient air, might act to functionally remove HIF1α signaling from the processing of environmental signals necessary to develop a normal immunity. The data from the hyperoxic neonatal exposures indicates that HIF1α levels are indeed decreased after being exposed to 75% O_2 from PN0-PN14. Functionally, this would mirror the results demonstrated in the PN4 HIF1α-deficient mice. In fact,

small molecule-induced stimulation of the HIF1 signaling cascade during hyperoxia can alleviate some of the structural deficits induced by neonatal hyperoxia exposure [118]. Clinicians, therefore, might explore the relationship between oxygenation, HIF1α-mediated changes in inflammation, and susceptibility to chronic lung disease in preterm infants [119, 120]. It should be noted that the link between preterm birth and changes in allergen-induced inflammation might only be beneficial in certain conditions. A recent report has demonstrated a decreased risk of allergic rhinitis is correlated with a low gestational age at birth [121].

Recently, there is increasing evidence that HIFs have an important regulatory role in inflammation. Additionally, besides the typical oxygen-dependent pathways that HIFs can be activated, it has been shown that HIFs can also be induced by inflammatory cytokines, though the mechanism of this induction is not completely understood. Pro-inflammatory cytokines like TNFα and IL1β have been shown to increase both the accumulation and transcriptional activity of HIF1α [122]. Since HIFs can be activated in response to inflammatory cytokines, this hints that HIF may play an important and complex role in inflammation [123]. IL1R2 blocks the processing of IL1β propeptide and binds mature polypeptide, inhibiting its ability to bind IL1R1. IL1R2 acts as a decoy receptor for circulating IL1α and IL1β, inhibiting them from binding IL1R1 to further the signaling cascade. The loss of IL1R2 expression should then increase the levels of IL1β [71]. A study focused on the role of galectin-3 in an OVA model [67]. In this study it was shown that peribronchial inflammatory cells had increased expression of galectin-3. BALF from OVA-challenged animals also had significantly higher amounts of galectin-3 present than in the BALF of control mice. Additionally, it was

noted that OVA-sensitized and challenged galectin-3 null mice had fewer eosinophils, less goblet cell metaplasia and lower airway resistance than wildtype controls. These galectin-3 null mice also had a decreased Th2 response with an increase in Th1 response, indicating that galectin-3 may regulate the switching to and from Th1 and Th2 responses. The immunohistochemistry from galectin-3 and IL-1R2 gives some indication as to potential epithelial-derived signaling factors that may be responsible for linking the loss of HIF1α specifically from the epithelium and a modulation of immunity. Similar staining was seen in DOX treated HIF1α-deficient mice, as well as in hyperoxia treated mice, potentially indicating that similar pathways are involved in the two models. It can be hypothesized that the loss of HIF1α, specifically from the epithelial cells of the lung could, in turn, alter the signals coming from those epithelial cells which aid in creating the atmosphere to direct the type of inflammatory response. If this occurs at a particularly important time period for HIF1α signaling, for example during post-natal lung development, it could potentially result in altered signaling that may reprogram the immune response in the lung.

In addition to the flow cytometry experiment presented, experiments were also performed after the mice were put through the OVA protocol previously described. The difficultly that arose was that the phenotype of the HIF1α-deficient mice is one of degrees. Both the HIF1α-sufficient and HIF1α-deficient mice have eosinophilia, just the latter is to a higher degree. Because of this, and also because the DC populations are so small to begin with, changes of less than 1% between DC populations were observed. In order to better differentiate the subpopulations, the cobalt-induced lung injury model was used. This made determining DC subpopulations more apparent

since the phenotype in that model is a switch from neutrophilia to eosinophilia, not just an exacerbated response.

In summary, the results from our current study indicate that HIF1α plays a role in programming the ability of the immune system to respond to insults, particularly aerosolized allergens. Only when HIF1α is deleted from the epithelial cells of the lung in a narrow window postnatally (PN4-14) is an exacerbation of the Th2 mediated response observed. Other studies have implicated neonatal hyperoxia in affecting the programming of the immune system, which implies that because HIF1α is responsive to oxygen, low levels of HIF1α may have this same effect, as would the mice in our study that lack HIF1α in their lung epithelial cells. Further investigation into the way in which the immune system is altered and the signaling involved in this change is needed to determine a mechanism for this alteration.

CHAPTER FIVE

Summary and Conclusions

5.1 The role of HIFs in allergic airway inflammation

When studying the role of HIF1α in allergic airway inflammation by using epithelial, lung-specific, inducible knockout mice, it was determined that losing HIF1α early in life (PN4-PN28), before the post natal development of the lung was completed was responsible for the greatest exacerbation in phenotype. These mice had increased total cells in their BALF, specifically increased numbers of eosinophils. These mice also had increased cellular infiltrates as viewed by H&E staining of histological samples, as well as increases in MBP staining, indicative of eosinophils, via immunohistochemistry. In addition to cellular characteristics of exacerbation, the HIF1α-deficient mice treated with OVA also had significantly increased total lung resistance as measured with the flexiVent protocol. When loss of HIF1α was induced from PN32-42, a window of time after post natal development of the lung is complete; the HIF1α-deficient mice looked very similar to controls.

In contrast to the HIF1α-deficient mice, loss of HIF2$\alpha^{fl/fl}$ mice during the post-natal developmental window (PN4-PN28 DOX dosing scheme), displayed no change in phenotype upon OVA sensitization and challenge. This lack of response was not due to lack of recombination as immunohistochemistry demonstrated significant reduction in HIF2α levels upon DOX exposure. Despite this loss, HIF2α-sufficient mice and HIF2α-deficient mice looked indistinguishable from controls. This held true for BALF cellularity, H&E and MBP histology, as well as functional analysis of total lung resistance. It should be noted that the HIF2α-deficient, OVA treated group in the analysis of total lung

resistance did seem to trend a little lower than the HIF2α-sufficient, OVA treated mice. Perhaps with an increased number of replicates this trend may become significant.

Finally, when using the HIF1/2α$^{fl/fl}$ mice in the OVA protocol using the PN4-PN28, they seemed to generally be protected from the signs of allergic airways. The OVA treated HIF1/2α-deficient mice had decreased in total cells in their BALF, specifically eosinophils, as well as decreases in cellular infiltrates as seen in H&E histology. Additionally, MBP staining via immunohistochemistry seemed to be decreased in OVA treated HIF1/2α-deficient mice when compared to control mice treated with OVA. Finally, using the *flexiVent* protocol to test methacholine-induced total lung resistance showed a trend that the HIF1/2α-deficient mice had lower total lung resistance than the OVA treated HIF1/2α-sufficient controls.

The biggest conclusion from these studies is that HIF1α has a role, particularly in the first 2 weeks of life (PN4-14), that has an influence on the immune system and the ability of the mouse to produce a controlled Th2-mediated response to OVA. The data from the HIF1/2α-deficient mice showing a decreased response to OVA also show that it is perhaps not just the HIF1α levels, but an adequate balance between HIF1α and HIF2α that is required for an appropriate inflammatory response.

5.2 Hyperoxia, prematurity and epithelial-derived signaling

Experiments involving hyperoxia exposure from PN0-PN14 showed a similar exacerbation of total cells, specifically eosinophils in the BALF after OVA sensitization

and challenge later in life. This is similar to the epidemiological studies that have linked premature birth with an increased incidence of asthma and allergy later in life [84, 94]. In addition, immunohistochemistry on the lungs of these mice showed increases in Galectin-3 as well as IL-1R2, common epithelial-derived signaling molecules that are known to modulate immunity. Since these molecules are epithelial-derived, they could serve to link the epithelial-specific loss of HIF1α in the mouse model with changes in inflammatory response.

5.3 Future work

The ATII and Clara cells are such a small percentage of the total cells of the lung, it makes getting meaningful data from a whole tissue nearly impossible. One important experiment that needs to be completed therefore is the careful isolation and biochemical analysis of ATII and Clara cells from control and HIF1α-deficient mice. The *in vivo* data has been collected, but more cell-specific data will next come from *in vitro* culturing of ATII cells and co-culturing experiments with other cell lines (or primary isolated cells) like dendritic cells or alveolar macrophages.

Also, it has been recommended that the serum from all of these animal experiments be tested for IgG and IgE levels. These samples already exist, so they should be processed. These data will be especially useful in confirming the difference between the 'lack of a response' and 'protection' in the HIF1/2α-deficient experiments. If proper levels of IgG and IgE are present, it can be concluded that the HIF1/2α-

deficient mice really were protected from Th2-mediated inflammation and that the lack of response isn't because they were not properly sensitized and/or challenged.

The results from the hyperoxia experiments can also be used to take further steps in understanding the relationships between premature birth, oxygen supplementation, changes in HIF signaling and alterations in immune response. One interesting idea is to attempt to reverse the effects of hyperoxia treatment by dosing with the PHD inhibitor DMOG. Dosing with the inhibitor should allow for HIF1α signaling to continue as needed for lung development, despite the excess of oxygen. Also, it is important to determine if hyperoxia-induced Th2 bias can be separated from hyperoxia-induced alveolar simplification. To test this concept, the hyperoxia experiments could be repeated with various levels of oxygen. Slightly lower, but still hyperoxic, levels of oxygen have been shown to minimize hyperoxic-induced alveolar simplification as well as decreases in surfactant production. Hopefully it will be possible to recreate the Th2 bias while maintaining the intgegrity of lung development at one of the lower hyperoxia levels. Hyperoxia experiments can also be performed with and without DOX, therefore with and without HIF1α specifically in the epithelial cells of the lung. These experiments would help to dissect the specific location of loss of HIF1α and therefore attempt to determine direct involvement of HIFs in hyperoxia effects.

After all of this data and experimentation, it is useful to propose a model of how the lack of HIFs in ATII and Clara cells can affect the immunity of the lung. In the alveoli of wildtype animals which have resident macrophages and ATII cells that express HIF1α. In this case, it is proposed that the signaling crosstalk between the HIF1α-

sufficient ATII cells and the resident macrophages results in the development of a particular kind of DC, hypothetically called an hDC for 'HIF1α-DC'. These hDCs are able to recruit and direct both Th1 and Th2 inflammatory responses. In the case where the ATII cells do not have HIF1α expression, the epithelial derived signaling molecules, perhaps like galectin-3 and IL1R2, differ from the previous situation, resulting in the development of a slightly different flavor of DCs, hypothetically called an nhDC for 'no-HIF1α-DC'. It is this slightly different type of nhDC that is able to only promote and direct Th2-mediated inflammation.

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